Mechanisms of Epithelial Branching, Nephrogenesis, and the Role of the Rho-GTPase Family in Kidney Development

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To Mina

Table of contents

Declaration

I declare that:

This thesis has been composed by myself.

The work presented here is my own, except where stated otherwise.

This work has not been submitted for any other degree or professional qualification.

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Nils Olof Lindström

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List of Abbreviations

α	Alpha
aPKC	Atypical protein kinase C
Arp2/3	Actin related protein 2/3
β	Beta
BC	Bowman's capsule
BDM	2,3 butanedione monoxime
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
Brn1/Pou3f3	Brn1/ POU transcription factor class 3
BS	Bowman's space
c-Met	Receptor tyrosine kinase c-Met
c-Ret	Receptor tyrosine kinase c-Ret
CamKII	calcium/calmodulin dependant kinase II
CB	Comma-shaped body
Cdc42	Cell division cycle 42
Cdk	Cyclin-dependent kinase
cDNA	complementary DNA
CKCM	Complete kidney culture medium
CM	Cap mesenchyme
CO_2	Carbon dioxide
Cre	Cre-recombinase
CSB	Comma- and S-shaped body
DBA	Dolichos biflorus agglutinin (lectin)
°	Degree
Dkk1	Dickkopf homologue 1
DT	Distal tubule

E	Embryonic day
EDTA	Ethylene diamine tetraacetic acid
EF2	Transcription factor protein
Erk/MAPK	Extracellular regulated MAPK/Mitogen-activated protein kinase
ERM	Ezrin, radixin and moesin proteins
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Flox	Encompassed by loxP sites
G0	G0-phase/Quiescent phase
G1	Growth-phase 1
G2	Growth-phase 2
GAP	GTPase activating protein
GDNF	Glial cell-line derived neurotrophic factor
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
gp135	glycoprotein 135
Greb1	Gene regulated by oestrogen in breast cancer protein 1
GSK3β	Glycogen synthase kinase 3 Beta
GTP	Guanine triphosphate
H1152	glycyl-H1152 dihydrochloride
H3(p)	Phosphorylated histone 3 (Ser10)
HEK293	Human embryonic kidney 293
Hes5	Hairy and enhancer of split 5
Hey1	Hairy/enhancer-of-split related with YRPW motif 1
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
Hox	Homeobox gene family
‘hrs’	Hours
HSC/P	Haematopoietic stem cell/progenitor

Hunk/MAK-V	Hormonally upregulated neu-associated kinase/MAK-V
HUVEC	Human umbilical vein endothelial cell
IC50	Inhibitor concentration 50%
IP3	inositol-1,4,5-triphosphate
Irx3	Iroquois related homeobox 3
Jag1	Jagged 1
JNK	Jun N-terminal Kinase
Lim1/Lhx1	LIM homeobox 1
LIMK	Lin11, Isl1 and Mec3 Kinase
LRP5/6	Low density lipoprotein receptor-related protein 5/6
µ	Micro
m	Milli
M	Mitosis
MDCK	Madin-Darby Canine Kidney
mDia	Mouse diaphanous protein homologue 1
MEK1	MAPK kinase kinase 1
MKK	MAPK kinase kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MM	Metanephric mesenchyme
$MnCl_2$	Manganese chloride
MTX	Methotrexate
NCAM	Neural cell adhesion molecule
NFAT	Nuclear factors of activated T cells
NHE1	sodium-hydrogen exchanger 1
NIH3T3	National Institutes of Health 3T3
PAK	p21-activated kinase
Par	Partitioning defective gene
Pax2	Paired box 2

PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Parietal epithelium
PFA	Paraformaldehyde
PKN	Protein kinase
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol (3,4)-diphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PLC	Phospholipase C
PT	Proximal tubule
PTEN	Phosphatase and tensin homologue
Rac1	Rac1-GTPase
Rb	Retinoblastoma tumour suppressor protein
RhoA	RhoA-GTPase
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK-I/II	Rho-kinase I/II
RT	Room temperature
RV	Renal vesicle
S	DNA-synthesis phase
Sall1	Sal-like 1
SB	S-shaped body
SB+	S-shaped body +
SEM	Standard error of the mean
Sglt2/Slc5a2	Sodium glucose transporter 2/solute carrier family 5 member 2
siRNA	Small interfering RNA
Six2	sine oculis-related homeobox 2
Sox9	sex-determining region Y (SRY) box 9
TAK1	Transforming growth factor β1 activating kinase 1

Tiam1	T-cell lymphoma invasion and metastasis 1
TCF/ LEF	Transcription factors
TGFβ	Transforming growth factor β1
THP	Tamm-Horsfall protein
TRITC	Tetramethyl rhodamine isothiocyanate
UB	Ureteric bud
Vangl2	van Gogh-like 2
VC	Vascular cleft/glomerular cleft
VE	Visceral epithelium
WAVE	WASP family Verproline homologous proteins
Wnt	Wingless/Int
WT1	Wilms' tumour 1 homologue
ZO-1	Zona occludens 1

Nomenclature

This thesis follows standard scientific nomenclature for genes, proteins, and conditions. Genes are specified in italics and proteins are in standard text. Where a gene and its protein are discussed jointly, the one with the main focus takes precedence in terms of the font used.

Abstract

The metanephric kidney consists of two types of epithelia; the Wolffian duct-derived ureteric bud and the nephrogenic components that originate from mesenchymal-to-epithelial transitions in the metanephric mesenchyme. The ureteric bud forms when inductive signals from the metanephric mesenchyme stimulates the evagination of an epithelial tube from the Wolffian duct into the mesenchyme. Reciprocal signalling between the ureteric bud and the metanephric mesenchyme regulates the branching of the ureteric bud and the induction of nephron formation. Inductive and inhibitory signalling of ureteric bud growth and branching has been shown by several protein families, however, the mechanical aspects of ureteric bud branching and nephrogenesis are largely unknown.

I investigated the roles of Rac1-GTPase and Rho-kinase during kidney development. These proteins are important regulators of the cytoskeleton where Rac1 is a promoter of actin filament polymerisation and Rho-kinase directly stimulates the formation and contraction of actin-myosin stress fibres. Using a cell-permeable inhibitor, Rac1 was inhibited with no effects on nephron formation or subsequent segmentation and patterning. Inhibition of active Rac1 significantly reduced the level of ureteric bud branching and also resulted in lower proliferation rates.

Rho-kinase was similarly targeted using two inhibitors. Rho-kinase inhibition had important effects on nephron formation and nephron maturation. Inhibition of Rho-kinase resulted in decreased levels of nephron formation and severely morphologically abnormal nephrons. The formation of apical-basal polarity was disturbed as was the development of the visceral and parietal epithelia; precursors of the renal corpuscle. Inhibition of Rho-kinase led to abnormal formation of the proximal-distal axis and abnormal segmentation of the nephron.

The effects of Rho-kinase inhibition were partially mimicked by direct targeting of actin-myosin contractions using a myosin-ATPase inhibitor. This demonstrated that Rho-kinase is necessary during multiple stages of nephrogenesis and maturation, at least in part, as a result of its ability to regulate actin-myosin contraction.

These results show that Rac1 and Rho-kinase play important roles during several aspects of kidney development and highlights the significance of further investigating the mechanisms involved during kidney organogenesis.

Chapter 1

Introduction to mechanisms of kidney development, nephron development, Rac1-GTPase and RhoA-GTPase

1.1 Overview

The focus of this thesis is on the development of the metanephric kidney and specifically the roles of Rac1-GTPase and Rho-kinase. These proteins have each been implicated as necessary components of normal kidney development (Michael, Sweeney, and Davies 2005; Meyer et al 2006; Osafune et al 2006). Rac1-GTPase (Rac1) and Rho-kinase (ROCK) are both integral for the regulation of planar cell polarity and for the control of actin and actin-myosin cytoskeletal components. In this thesis I present data indicating that normal kidney growth, ureteric bud branching, and proliferation are processes dependent on Rac1 activity. Nephron formation, apicobasal polarity formation, glomerular cleft development and proximal-distal nephron patterning show a requirement for ROCK.

The development of the kidney, like all other organs, can be discussed at different levels of resolution. The complexity of the kidney renders it impossible to cover completely in a single piece of work, thus various efforts have been made in order to describe the kidney in detail from several different points of view. From an anatomical/physiological point of view, *Brenner & Rector's, The Kidney, 6th Ed.* (Brenner 1999) covers the human kidney in great detail. From an anatomical/developmental perspective, the work *Organogenesis of the Kidney* (Saxen 1987) discusses normal kidney development and *The Kidney: From Normal Development to Congenital Disease* (Vize, Woolf, and Bard 2003) provides excellent insights into the development and the abnormalities found in the mouse kidney. For an ontology of the kidney from development through to adulthood, Little et al 2007 offers a detailed, up-to-date description of all known components in the kidney (Little et al 2007).

In order to introduce the topic of kidney development efficiently, I will discuss the mechanisms of morphogenesis under several sub-headings, each with the appropriate level of detail. This has been done because there are several different compartments within the kidney that not only represent different cellular lineages, such as the metanephric mesenchyme and the ureteric bud, but they also differ in the processes that take place within them. This chapter will start by introducing the kidney in terms

of its importance for homeostasis I will then cover the origin of its compartments where the development of the ureteric bud and the nephrons will be discussed in detail. Particular focus will be placed on the stages and mechanisms of ureteric bud branching, nephrogenesis and nephron maturation. Where appropriate, Rac1 and ROCK will be discussed in terms of their possible, proven or closely linked roles to kidney development. However, as they carry out important functions in a large number of systems, the importance of each protein will be discussed mainly within the results chapters as the relevance of their functions appear.

1.2 The kidney and its origins

1.2.1 The functional importance of the kidney

The kidney performs three main functions: (i) to separate wanted and unwanted compounds carried by the blood and (ii) to regulate the removal of excess water from the blood, and (iii) to recapture water from the urine (Dickinson et al 2007). The kidney performs its roles by acting as a filtration apparatus between the visceral components (blood vessels) and the tubular systems (nephrons/collecting ducts in the kidney) and by being highly capable of reabsorbing selected ions and small solutes. The actual filtration unit of the kidney is the renal corpuscle, which is a small capsule-like portion of the nephron. The renal corpuscle permits the very close interaction between a double-layered and complex basement membrane and the blood. This basement membrane, together with a highly adapted and unique cell type (the podocytes), makes up the filter that separates the blood from the epithelial tubule's lumen, which eventually leads to the bladder and away from the body. The blood is supplied to the kidney from the aorta via the renal artery which through a series of subdivisions becomes the afferent arteriole which enters the glomerulus in the renal corpuscle (Brenner 1999). Although the flow of metabolites and fluid from the blood through the glomerulus is effective, the filtration is somewhat non-selective in that some desired molecules such as salts and water are also permitted to pass through the filtration. The kidney's ability to reabsorb such molecules is

adjustable and thus provides it with the means to regulate salt concentrations and water excretion for homeostatic purposes.

1.2.2 The origin of the kidney

The epithelial components within the metanephric kidney, the collecting duct system and the nephrons, both originate from the intermediate mesoderm, as do all the epithelial kidney structures. The developmental stages that occur between the specification of the lineages in the intermediate mesoderm and the final stage when the kidney is formed, differ for the two types of epithelia. The collecting duct epithelium in the kidney originates from the nephric duct/Wolffian duct whereas the nephrons originate from the most caudal part of the nephric cord, the metanephric mesenchyme, reviewed by Saxen 1987. During development, the Wolffian duct grows in a rostral to caudal direction, adjacent to the nephric cord where it, together with the nephric cord, forms three sets of kidneys. Prior to the development of the metanephric/permanent kidney, two sets of transient kidney structures are formed, the pronephric kidney around embryonic day 8 (E8) and the mesonephric kidney at E9.5, reviewed by Saxen 1987. The pronephric and the mesonephric kidney are ephemeral structures in the mouse although the mesonephric kidney contributes to the efferent ducts and the vas deference in male animals, discussed in Saxen 1987. The pronephric and the mesonephric kidneys do not contribute to the final structures for blood filtration and excretion which is carried out by the metanephric kidney. The metanephric kidney forms when the Wolffian duct has elongated to a point perpendicular to the dorsal hind limbs at around E10-E10.5. Inductive signalling by glial cell-line derived neurotrophic factor (GDNF), from the adjacent medial metanephric mesenchyme, results in a small ureteric bud evaginating, elongating, penetrating and branching within the metanephric mesenchyme (Vega et al 1996; Sanchez et al 1996; Pichel et al 1996; Sainio et al 1997). Other genes such as transcription factors *Sal-like 1* (*Sall1*) and *Wilms' Tumour 1 homologue* (*WT1*) are also necessary for ureteric bud invasion and in knockouts this process fails to occur (Kreidberg et al 1993; Nishinakamura et al 2001). Through reciprocal signalling, the

ureteric bud activates the canonical Wnt-signalling pathway in the metanephric mesenchyme, possibly by secretion of Wingless/Ints 9b (Wnt9b), which has been shown to stimulate nephron formation (Carroll et al 2005; Park, Valerius, and McMahon 2007). These processes set the stage for the main epithelial morphogenetic processes that take place during kidney development and each process will be discussed in further detail below.

1.3 The collecting duct system in the kidney

1.3.1 The ureteric bud

The collecting duct system is the mature structure that is derived from the embryonic ureteric bud which, as previously mentioned, forms by evagination from the Wolffian duct. This form of epitheliogenesis, where new epithelia forms by outgrowth from already existing epithelia, is relatively common during development and can be seen for example in the lung, prostate, and mammary glands; for reviews of their development see Warburton and colleagues, Timms, and Watson and Khaled (Warburton et al 2000; Timms 2008; Watson and Khaled 2008). The mechanisms that regulate the formation and the branching of the ureteric bud are largely unknown but some circumstantial evidence is beginning to emerge. The initial consideration is to understand whether the mode of ureteric bud branching lies towards producing a stereotypically branched structure or if the branching is a more plastic and inducible process. In the extreme sense this can be considered as the divide between an autonomous genetically stereotyped programme, versus branching morphogenesis that would result from the induction and inhibition by environmental factors. Early lung development, for example, produces a relatively stereotypic left-right asymmetrical pattern which is a result of the early development of the left-right body axis programming, as reviewed by Cardoso and Lu (Cardoso and Lu 2006). Drosophila tracheal development has also been shown to be regulated by several genes that produce a highly replicable branching pattern through time and space where tubule diameter, length, and branching are all determined by a genetic

programme, as shown and discussed by Beitel and Krasnow (Beitel and Krasnow 2000). Superficially, normal ureteric bud formation and early development also follows what appears to be a fairly reproducible pattern. Ureteric bud evagination occurs at a highly replicable location just before E11 and forms the characteristic, single-branched, t-bud stage at E11.5 (Saxen 1987). However, the induction and branching of the ureteric bud is dependent on signalling molecules such as GDNF that are expressed in the metanephric mesenchyme and which are detected by receptors expressed by the ureteric bud, as shown and discussed by Sainio and colleagues (Sainio et al 1997). Growth factors can both increase (e.g. FGF7) (Qiao et al 1999) and decrease (e.g. BMP2) (Piscione et al 1997) branching as well as produce ectopic bud formation or branching in regions normally unbranched (e.g. GDNF) (Sainio et al 1997). This suggests that the branching morphogenesis of the ureteric bud is strongly modulated by its environment perhaps suggesting that the emphasis lies towards providing a mechanism of branching that is highly plastic. Some components of ureteric bud branching can however be considered as adhering to a genetic programme in the sense that the ureteric bud is a result of a number of events that predispose it to behave in a certain manner.

1.3.2 Mechanisms of branching in the ureteric bud

Several different mechanisms could act alone, or in unison, to regulate the development of the collecting duct system. It is important to understand how branching of the ureteric bud occurs since it will produce the shape of the mature collecting duct and also because of its close interaction with, and ability to induce, the metanephric mesenchyme. The pattern of ureteric bud branching ultimately leads to the pattern by which the nephrons will form. In this section I review some of the processes that are known, or considered likely, to be involved in the regulation of the ureteric bud morphogenesis.

The evagination of the ureteric bud from the Wolffian duct represents the first of many branching events that the ureteric bud undergoes to create the final collecting

duct system. As discussed, numerous proteins have been shown to be part of the branching regulatory pathway in the ureteric bud. Extracellular signalling proteins FGF7 and FGF10, reviewed by Bates (Bates 2007), GDNF (Vega et al 1996), and Pleiotrophin (Sakural, Bush, and Nigam 2001), and the intracellular signalling protein Sprouty (Chi et al 2004) are examples of such proteins. The discovery of growth factors that stimulate or inhibit growth has shown a lot about how the ureteric bud develops but it has shown significantly less about what mechanisms produce the branching and growth. Trying to elucidate how the ureteric bud branch is a typical trap-door problem (Davies 2005). Like a trap-door, the development of a shape or a cell lineage is mainly unidirectional. The main type of data that can be obtained from a standard experiment will represent the kidney at that time-point, and it is tricky to determine how a branch came to be or how a nephron shape formed, as discussed by Srinivas and colleagues (Srinivas et al 1999). The development of a *homeobox gene family 7b (Hox7b)-GFP* mouse solved the problem to determine the mode of how the ureteric bud branches, at least on a macroscopic level (Srinivas et al 1999). Using *Hoxb7-GFP* mice allowed for detailed time-lapse video recording of branching ureteric buds in cultures (Watanabe and Costantini 2004). The authors showed that 75% of all branching during the first 7 set of branching events occur by terminal bifurcations of the ureteric bud. That means that the vast majority of branching occurs at the most terminal points of the ureteric bud and each ureteric bud divides into two sub-branches. The remaining 25% of branching events took place by either terminal trifurcations (dividing into three), 18%, or by lateral branching, 6% (Watanabe and Costantini 2004). It worthwhile to keep in mind that it is possible that the mode of ureteric branching could change after the first 7 branch generations which is how long the authors investigated ureteric bud branching. In addition, the mode of branching that was detected in the culture conditions used by the authors could differ from how the ureteric bud normally branches in vivo. In the mammalian lung, the mode of branching alters after around 2 generations in order to continue by the process of clefting, as reviewed by Warburton (Warburton et al 2000).

One possible mechanism for the growth of the ureteric bud, as well as for its branching, is proliferation. The growth of the ureteric bud has been suggested to

occur by the relatively rapid proliferation rates seen in the ureteric bud tips (Michael and Davies 2004). The mammary glands, another highly branched epithelial structure, have been shown to be dependent on proliferation for normal duct initiation (Ewald et al 2008), as has the ureteric bud (Michael and Davies 2004). Other recent evidence now adds additional support to the hypothesis that proliferation might be important for branching as was shown using mice mosaic for *c-Ret*$^{-/-}$*/Hoxb7-GFP* cells and wild type cells (Shakya, Watanabe, and Costantini 2005). *C-ret*, the GDNF receptor, is normally detected expressed in the ureteric bud tips, where one of the GDNF/c-ret functions appears to be the regulation of proliferation (Michael and Davies 2004; Shakya, Watanabe, and Costantini 2005). In the mosaic mice, *c-ret* deficient GFP labelled cells failed to contribute to the ureteric bud tips and displayed gradually reducing numbers towards branches formed during later generations of branching events. This inability of *c-ret* deficient cells to contribute to the tip regions might have resulted from them not expressing tip-like proliferation rates and therefore becoming a gradually smaller fraction of the total number of cells in the ureteric bud (Shakya, Watanabe, and Costantini 2005). This reinforces the suggestion that the majority of proliferation occurs in the ureteric bud tips and that this is accountable for the main proportion of growth displayed by the ureteric bud. However, this does not prove that proliferation rates are directly linked to the process of branching although the inhibition of proliferation results in reduced ureteric bud emergence and reduced branching (Michael and Davies 2004) and (this work).

Cellular migration is another important process during embryogenesis. The possibility of motility acting as a morphogenetic mechanism in the ureteric bud would be attractive as it could provide insights into how tubular branching occurs. If cells at the ureteric bud tips are subjected to and are responsive to chemotactic signals then individual cells, or cells moving as a sheet or in a group, could force morphological changes in the epithelium which could lead to new branches forming. In addition, as motility can be both rapidly induced and inhibited this could provide a mechanism for how the spatial patterning of the ureteric bud tree is regulated. This would have to be highly regulated in order to affect only those regions of the ureteric

bud tips that are required to produce new branches. The directed migration of a single cell or a sheet of epithelial cells have been shown to be potent mechanisms for epithelial reorganisation in for example wound healing (Desai et al 2004) as well as during mammary gland branching (Ewald et al 2008). The metanephric mesenchyme is known to express *Hepatocyte growth factor/Scatter factor (HGF/SF)* (Woolf et al 1995), where the HGF protein is a potent motogenic factor for epithelial cells in vitro (Stoker et al 1987; Weidner et al 1991). The HGF receptor, *c-met* has also been shown to be expressed by the ureteric bud (Woolf et al 1995). Interestingly, studies of HGF in the kidney have resulted in contradicting conclusions. Using an HGF antibody-inhibitor approach, Woolf and colleagues showed that kidney rudiment cultures exhibited reduced branching and nephrogenesis as well as increased apoptosis (Woolf et al 1995). In the *HGF* null mutant mouse on the other hand, the kidneys developed normally (Schmidt et al 1995).

More direct evidence has also suggested that epithelial cells in the ureteric bud tips are motile. Using the *Hoxb7-GFP* reporter mice previously described, Watanabe and Costantini showed that cells in the ureteric bud tips display changes in shape and orientation in a very dynamic manner that is reminiscent of motile cells (Watanabe and Costantini 2004). Not only do the cells appear to be motile but a study looking at the importance of the cytoskeleton in the kidney showed that the mild disruption of actin filaments results in morphologically aberrant ureteric bud tips displaying what appears as scattering of epithelial cells from the ureteric bud tips (Michael, Sweeney, and Davies 2005). This was suggested to perhaps reflect a motogenic mechanism in the ureteric bud tips. The authors speculated that the disruption of actin might result primarily in the weakening of already-loose cellular junctions in the ureteric bud tips and that the mechanisms of motility, which demand only very short-lived and short actin strands, remained functional (Michael, Sweeney, and Davies 2005).

Kim and Dressler directly claimed that cellular motility is involved during renal branching morphogenesis (Kim and Dressler 2007). The authors studied the protein Phosphatase and tensin homolog (PTEN). PTEN has been shown to be a negative regulator of cell motility which by reciprocal actions and localisation pattern counteracts the motogenic effects of Phosphatidylinositol 3-kinase (PI3K), as was studied in Dictyostelium (Funamoto et al 2002). PI3K phosphorylates

phosphatidylinositol (3,4)-diphosphate (PIP2) into the motogenic form, phosphatidylinosotol (3,4,5)-triphosphate (PIP3), whereas PTEN acts as a PIP3 phosphatase, as reviewed by both Funamoto and colleagues, and Kim and Dressler (Funamoto et al 2002; Kim and Dressler 2007). Firstly, Kim and Dressler illustrated that Madin-Darby Canine Kidney (MDCK) cells, expressing a mutant form of the *pten* gene, showed increased chemotactic responsiveness to GDNF, whereas cells over expressing the wild type *pten* gene exhibited no chemotactic response to GDNF (Kim and Dressler 2007). Secondly, the authors combined the floxed mutant *pten* gene with a *hoxb7-Cre* gene with the result of producing mice with *pten* negative ureteric buds. The resulting ureteric buds displayed abnormally shaped tips with irregular basement membranes. Due to the results obtained from the MDCK cells, the authors interpreted these results as evidence that a mechanism of cell migration is present during ureteric bud branching morphogenesis and the disruption of this, leads to the aberrant ureteric bud tips. However in an earlier publication, Martin-Belmonte and colleagues revealed an alternative explanation for the abnormal phenotype observed in the *pten* negative ureteric buds (Martin-Belmonte et al 2007). Martin-Belmonte and colleagues used MDCK cysts to study the development of polarity during cyst development. Their findings show that repressing PTEN production or inhibiting existing PTEN resulted in abnormal formation of the apical surface of MDCK cysts (Martin-Belmonte et al 2007). The same result could be achieved by interfering with Annexin 2, cell division cycle 42 (Cdc42) and atypical protein kinase C (aPKC) (Martin-Belmonte et al 2007). It was thus speculated that the localisation of PTEN to the apical surface of cells during cystogenesis, results in an apical accumulation of PIP2, which results in the recruitment of PIP2 binding protein Cdc42 (Martin-Belmonte et al 2007). Cdc42 binds aPKC/Par6, proteins known to be involved in the production of polarity (Martin-Belmonte et al 2007). Importantly, Rac1, a central protein in motility, regulation did not localise in a manner similar to Cdc42 which could suggest that their roles are different (Martin-Belmonte et al 2007). These results provide an interesting interpretation to the results obtained by Kim and Dressler in 2007 and demonstrates the importance to further elucidate whether motility plays a role during branching morphogenesis.

Epithelial morphogenesis can also be regulated by the contraction of actin-bands through processes that are not necessarily connected to migration. Actin-myosin contraction can lead to large-scale epithelial changes, as for example during Drosophila ventral furrow invagination (Nikolaidou and Barrett 2004; Fox and Peifer 2007). Another example of this type of morphogenesis can be found during the initiation of sea urchin gastrulation, where RhoA has been shown to be essential for the initial stages of invagination during archenteron formation (Beane, Gross, and Mcclay 2006). High amounts of apical actin have been demonstrated in the ureteric bud tips (Fisher et al 2001; Michael, Sweeney, and Davies 2005) as well as apical localisation of myosin (Meyer et al 2006). Interestingly, these actin bands disappear as a result of inhibition of both GDNF (Michael, Sweeney, and Davies 2005) as well as direct inhibition of the Erk/MAPK pathway (Fisher et al 2001). Branching morphogenesis in the kidney is highly disrupted by both the inhibition of actin-myosin contraction via inhibition of Rho-kinase as well as by direct inhibition of actin polymerisation (Michael, Sweeney, and Davies 2005). It is has not yet been determined whether these effects are a result of abnormal actin-myosin function in the ureteric bud tips.

Proliferation, migration and cytoskeletal rearrangements are some of the most favoured models for ureteric bud branching morphogenesis. There are other possible mechanisms such as, clefting and convergent extension, that could regulate branching and elongation. This is an area of kidney development that needs significantly more investment in terms of time and effort in order to provide a clearer picture of the machinery involved.

1.3.3 Ureteric bud branching and cellular differentiation

The branching of the ureteric bud occurs mainly by terminal bifurcation (Watanabe and Costantini 2004). The preference to branch at the ureteric bud tips does not necessarily mean that the ability to branch is restricted to these areas of the ureteric bud; it just shows where branching occurs. The ureteric bud tips express genes that

display a strongly tip-restricted expression pattern, *c-ret* (Pachnis, Mankoo, and Costantini 1993) as well as *Wnt11* and *sex-determining region Y (SRY) box 9 (Sox9)* are good examples, as reviewed by Michael, Sweeney and Davies (Michael, Sweeney, and Davies 2007). This regionalised expression of specific genes in the ureteric bud tips has previously been suggested to indicate that this is the region where the capability to branch is maintained (Michael, Sweeney, and Davies 2007). Significantly, this region correlates with the area previously defined to display elevated proliferation rates (Michael and Davies 2004; Michael, Sweeney, and Davies 2007). Recently a study was carried out to determine whether β-catenin might be important for the ureteric bud, considering the expression of numerous Wnts in the kidney. The ureteric bud expresses and is subjected to several Wnt proteins that could potentially signal through the canonical Wnt-signalling pathway, thus regulating the transduction of β-catenin signalling. The Wnt-signalling pathways will be described in further detail in this chapter in relation to nephron induction. This study crossed conditional *β-catenin* mutant mice with mice carrying the *Hoxb7-Cre*, thus removing *β-catenin* from the Wolffian duct and the ureteric bud (Marose et al 2008). These mice displayed some interesting phenotypes. The authors showed that the ureteric buds that invaded the metanephric mesenchyme failed to branch properly (Marose et al 2008). *β-catenin* mutant ureteric buds lost the expression of ureteric bud tip markers such as *Wnt11*, *Sox9* and *c-Ret*, and prematurely initiated expression of collecting duct/stalk maturation markers such as *zona occludens α1+* (*ZO1α1+*) and *Aquaporin 3* (Marose et al 2008). In addition, the expression of a non-degradable and stable form of β-catenin protein, in the ureteric bud, resulted in the failure of the ureteric buds to express maturation markers such as *Aquaporin 2* and *3* (Marose et al 2008). The authors thus accredited the abnormal branching to be a result of a failure in the ureteric bud to maintain the undifferentiated cell identity normally found in the ureteric bud tips (Marose et al 2008). Exactly which molecular mechanism it is that drives this tip-concentrated β-catenin signalling, is yet to be determined, however *Wnt11* is known to display tip-specific expression (Kispert et al 1996) and could be a potential regulator of β-catenin degradation. *Wnt11* mutants have been shown to have a disrupted branching morphology, although this has partially been attributed to reduced GDNF/c-ret signalling (Majumdar et al 2003).

A somewhat neglected, but equally possible, scenario is that a mutation of *β-catenin* might affect cell-cell junctions. β-catenin protein was originally considered, solely to be a component of adherens junctions where it forms a complex with α-catenin and E-cadherin and links the junctions to the cytoskeleton, reviewed by Niessen and Gottardi (Niessen and Gottardi 2008). It was only when it was discovered to be involved in Wnt-signalling that the focus somewhat shifted, as reviewed by Gavard and Mège (Gavard and Mege 2005). A loss of β-catenin protein could result in abnormal or failure of adherens junction formation since β-catenin has been shown not only to shield cadherins from degradation but also to provide stability through cytoskeleton linkage, reviewed by Niessen and Gottardi (Niessen and Gottardi 2008).

Interestingly, the ability of tip cells to differentiate in a tip-to-stalk direction has been shown to be bidirectional by demonstrating the ability of stalk cell to revert towards a tip cell identity (Sweeney, Lindström, and Davies 2008). These data are briefly explained here and a copy of the publication has been included in Appendix 1. These data are not presented as a results chapter since its focus is not directly in line with the theme of this thesis and because the data does not stand as a story without the data contributed by the other authors. In this article we have demonstrated that stalk regions of the ureteric bud, which have lost the expression of tip markers and differentiated into a stalk state, as shown by the gain of stalk markers, are capable of reverting from a ureteric bud stalk identity to the ureteric bud tip identity (Sweeney, Lindström, and Davies 2008). These reverted cells produce new tips that regain the expression of *Wnt11* were are able to undergo branching morphogenesis and induce nephron formation in a typical fashion (Sweeney, Lindström, and Davies 2008). The work by Marose and colleagues could provide a possible molecular mechanism for how the identity-reversal works (Marose et al 2008). A possibility is that β-catenin signalling might be important for the stalk-to-tip identity reversal as well as for the maintenance of the tip-identity. Future experiments should aim at determining whether the β-catenin signalling plays a role during the reversal of stalk cells into tip cells or if the β-catenin signalling is only initiated at a stage when this process has already begun. This would establish whether β-catenin signalling is a primary

regulator of tip-identity of if it is a downstream effector initiated as a response by the acquisition of a tip-identity.

1.4 The Nephrons

1.4.1 Nephron development: major stages and processes

The formation of nephrons occurs by a very different mechanism compared to that regulating ureteric bud induction. Nephrons form by the process of mesenchymal-to-epithelial transition. Mesenchymal-to-epithelial transitions are a rarer form of epitheliogenesis compared to that used for the formation of the ureteric bud, although they are seen in for example somitogenesis where each somite develops clear boundaries by a layer of cells transiting from a mesenchymal to epithelial state, as reviewed by Takahashi and colleagues (Takahashi et al 2005). A mesenchymal-to-epithelial transition entails a more fundamental reorganisation of the cells, compared to the evagination of a bud from already existing epithelia. The transiting mesenchymal cells are required to acquire an apicobasal polarity as well as to express new genes such as the structurally important protein cytokeratin and to develop junctional adhesion complexes. In addition, as a result of becoming epithelial, the cells deposit a basement membrane.

In this thesis, the development of the nephron is discussed in the order of the main structural stages through which each nephron progresses during development. Nephron formation is a sequential process and as a result, during development, new nephrons are continuously induced to form and mature. This means that, at any given time-point subsequent to the initial nephron induction, the kidney consists of an ordered pattern of nephrons at different developmental stages. The detail in which nephron induction and development is discussed herein is necessary because a large portion of the results in this thesis require an understanding of nephrogenesis.

1.4.2 Stage 1: Cap Mesenchyme

The cap mesenchyme is the structure containing the nephron progenitor cells. The nephron progenitor cells are nephron stem cells that possess the potential for self renewal as well as being able to produce a large number of different types of cells within the adult nephron (Kobayashi 2008). It is not clear when the progenitor cell population is defined and whether there can be recruitment from the metanephric mesenchyme into the cap mesenchyme once this population has formed. It is also not known if the cap mesenchyme is a homogenous population of cells of if sub-populations exist which might already be programmed for the specific contribution to particular nephron segments.

The term 'cap mesenchyme' refers to the mesenchymal cells directly surrounding the ureteric bud tips. The cap mesenchyme is characterised by the presence of several proteins, for example Neural Cell Adhesion Molecule (NCAM), Sine oculis-related homeobox 2 (Six2), Wilms' tumour 1 homologue (WT1) and Paired box 2 (Pax2) (Klein et al 1988a; Plachov et al 1990; Armstrong et al 1993; Self et al 2006). Structurally, the cap mesenchyme appears almost columnar and somewhat epithelial as is shown by the cells 'polarisation' towards the ureteric bud tips (see Fig.3.3 in Chapter 3). This might be a manifestation of the high amounts of adhesion molecules expressed by these cells such as NCAM (Klein et al 1988a). The cap mesenchyme contains nephron progenitor cells. *Six2* expression has been closely linked to this population of progenitors, as knockouts of *Six2* appear to result in the loss of the progenitor cell's ability to self-renew and the cells start to differentiate (Self et al 2006). Such *Six2* deficient progenitor cells do however maintain their ability to become induced and form nephrons and, in fact, ectopic nephron formation occurs surrounding the ureteric bud tips, which suggests that all the progenitor cells proceed toward a nephron fate (Self et al 2006). The closeness of the cap mesenchyme to the ureteric bud tips ensures that this population of mesenchymal cells is subjected to nephron inducing signalling molecules secreted by the ureteric bud, for example Wnt9b (Carroll et al 2005). Several key components have been identified that are important for the induction of nephron formation, for example,

Wnt4 and WT1 (Stark et al 1994; Davies et al 2004), also discussed by Park, Valerius and McMahon (Park, Valerius, and McMahon 2007). The first aspect of nephron induction that will be discussed, here is Wnt signalling, which in recent years has been shown to be essential for the initial steps of nephrogenesis.

1.4.3 Wnt signalling in the kidney

A large number of Wnt genes are expressed during kidney development, as shown below in Table 1.1. and in Diagram1.1 Wnt signalling can be divided into two main fractions: (1) the canonical/β-catenin-dependent pathway; (2) the non-canonical/β-catenin-independent pathway, as reviewed by Slusarski and Pelegri (Slusarski and Pelegri 2007), see Diagram1.2. Perhaps most important for this thesis, is the planar cell polarity which has been shown to act via Rho-kinase (Habas, Dawid, and He 2003). The importance of Wnt signalling for the kidney has been emerging for a long period of time and it appears that they play a pivotal role during kidney development and thus deserve a fairly detailed introduction.

Wnt	**Expression**	**Function**	**Reference**
Wnt2b	Stroma	Ureteric bud branching	(Lin et al 2001)
Wnt4	Nephrons	Nephron formation	(Stark et al 1994)
Wnt6	Ureteric bud stalk	Nephron induction	(Itaranta et al 2002)
Wnt7b	Mature collecting duct	Unknown	(Kispert et al 1996)
Wnt9b	Ureteric bud stalk	Nephron induction	(Carroll et al 2005)
Wnt11	Ureteric bud tip	Ureteric bud branching	(Kispert et al 1996; Majumdar et al 2003)

Table 1.1 Important Wnt genes expressed in the metanephric kidney.

A number of papers have begun to dissect out how the Wnt signalling pathways regulate kidney development (Park, Valerius, and McMahon 2007; Kuure et al 2007; Schmidt-Ott et al 2007; Schmidt-Ott and Barasch 2008; Marose et al 2008). The Wnt signalling pathways share a common element in that they all act through the trans-membrane receptor Frizzled. This is also where a divergence is seen. Through

activation of Frizzled, the canonical pathway continues by activating Dishevelled and inhibiting the β-catenin degradation complex; the non-canonical pathway splits into the Dishevelled-dependent activation of Rho pathways and Rac1-Jun N-terminal Kinase (JNK) pathways that regulate planar cell polarity as well as the Dishevelled-independent pathway that regulates calcium release and activates calcium dependent processes in addition to protein kinase C. For further information, see the reviews by Clevers for a description of the canonical pathway (Clevers 2006), Wang and Nathans for the planar cell polarity (Wang and Nathans 2007) and Slusarski and Pelegri for the calcium-dependent pathway (Slusarski and Pelegri 2007).

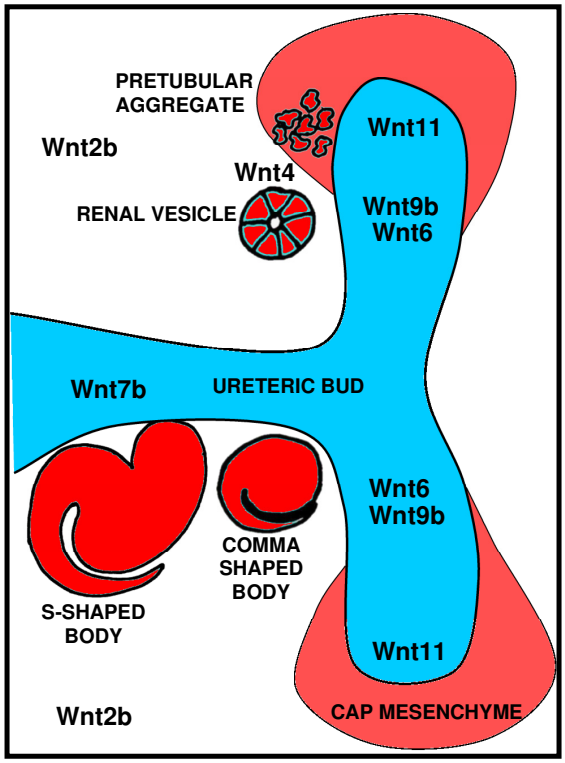


Diagram1.1 Wnt expression in relation to the embryonic kidney structures.

The diagram is based on references cited in the text.

1.4.4 Canonical Wnt signalling: the β-catenin pathway in the kidney.

The Wnt/β-catenin signalling pathway is known to regulate many cellular processes including, proliferation, cell survival and adhesion, as discussed by Marose and colleagues (Marose et al 2008). In the canonical Wnt pathway, Wnt binds to Frizzled and phosphorylated Dishevelled which in turn phosphorylates glycogen synthase kinase 3 Beta (GSK3β); this process leads to the recruitment of Axin to the Frizzled/low density lipoprotein receptor-related protein 5/6 (LRP5/6) membrane complex and as a result the disruption of the β-catenin destruction complex, as reviewed by Clevers, and by Schmidt-Ott and Barasch (Clevers 2006; Schmidt-Ott and Barasch 2008) The inhibition of the β-catenin degradation complex means that stable β-catenin can translocate to the nucleus and bind to members of the transcription factor family TCF/LEF, which regulates the cellular processes described here.

The β-catenin pathway has long been proposed to function in the kidney. Initial research showed that mimicry of Wnt signalling using GSK3β-inhibiting lithium ions, can drive nephron induction in culture (Davies and Garrod 1995). Since then, three major papers have demonstrated that the β-catenin signalling pathway is necessary and sufficient for nephron induction (Park, Valerius, and McMahon 2007; Kuure et al 2007; Schmidt-Ott et al 2007). These investigations demonstrated three major things. Firstly, the specific activation of β-catenin signalling using the expression of a stable mutant form of β-catenin in the nephron progenitor population of cells, as defined by *Six2* expression (Self et al 2006), results in the induction of these cells to become nephrons (Park, Valerius, and McMahon 2007; Kuure et al 2007). Secondly, the specific knockout of *β-catenin* in the same population of cells results in a complete loss of nephron induction (Park, Valerius, and McMahon 2007). Thirdly, during the transition period between un-induced cap mesenchyme and induced mesenchyme, a set of genes regulated by the β-catenin/TCF/LEF pathway is activated; a process that can be interrupted by the expression of a dominant negative form of TCF (Schmidt-Ott et al 2007). An interesting effect of the continuous activation of β-catenin signalling in the *Six2* positive progenitor cells, is that this effectively inhibits the transition from a mesenchymal to epithelial state (Park,

Valerius, and McMahon 2007). This shows that β-catenin signalling is a necessary process during the induction of nephron formation but is required to be reduced for the completion of nephron formation. This was previously shown by Davies and Garrod who demonstrated that the induction of nephrons using lithium was more efficient if lithium was only present during the initial 24 of culturing, although the connection with Wnt-signalling was not made (Davies and Garrod 1995). In order to further the understanding of β-catenin signalling two questions need immediate attention: (1) What activates the β-catenin signalling pathway in the progenitor cells? (2) What maintains the progenitor cell population? Likely candidates for the induction of the β-catenin signalling are Wnt9b and/or Wnt6 which have been shown to be expressed by the ureteric bud and are necessary and sufficient to inducing nephron formation (Itaranta et al 2002; Carroll et al 2005), see table 1.1. Not only do these Wnts induce nephron formation but their expression starts in the correct location on the tip/stalk border where nephron formation normally takes place (Itaranta et al 2002; Carroll et al 2005). A large number of Wnts have been demonstrated to be sufficient for induction of nephrogenesis. Wnt1, Wnt3a, Wnt4, Wnt7a and Wnt7b were all tested and shown to induce nephron formation by co-culturing of metanephric mesenchyme and Wnt expressing National Institutes of Health 3T3 (NIH3T3) cells (Kispert, Vainio, and McMahon 1998). It is noteworthy to mention that other proteins such as R-spondins are also capable of inducing the canonical β-catenin pathway, reviewed by Clevers (Clevers 2006). R-spondin 1 and 3 are both present in the metanephric mesenchyme during kidney development (Nam, Turcotte, and Yoon 2007).

As previously noted, it is also important to mention that β-catenin can also act as a component that is necessary for adherens junctions, reviewed by Gavard and Mège (Gavard and Mege 2005). In fact, it has been demonstrated that Wnt signalling can regulate a kidney specific cadherin, cadherin-6, in Madin-Darby Canine Kidney cells, a property that could have major impacts on nephron behaviour (Stewart, Barth, and Nelson 2000). It is possible that the necessity of β-catenin could very well be a result of abnormal adherens junctions, in particular since the nephron has been shown to express a large number of different cadherins (Dahl et al 2002).

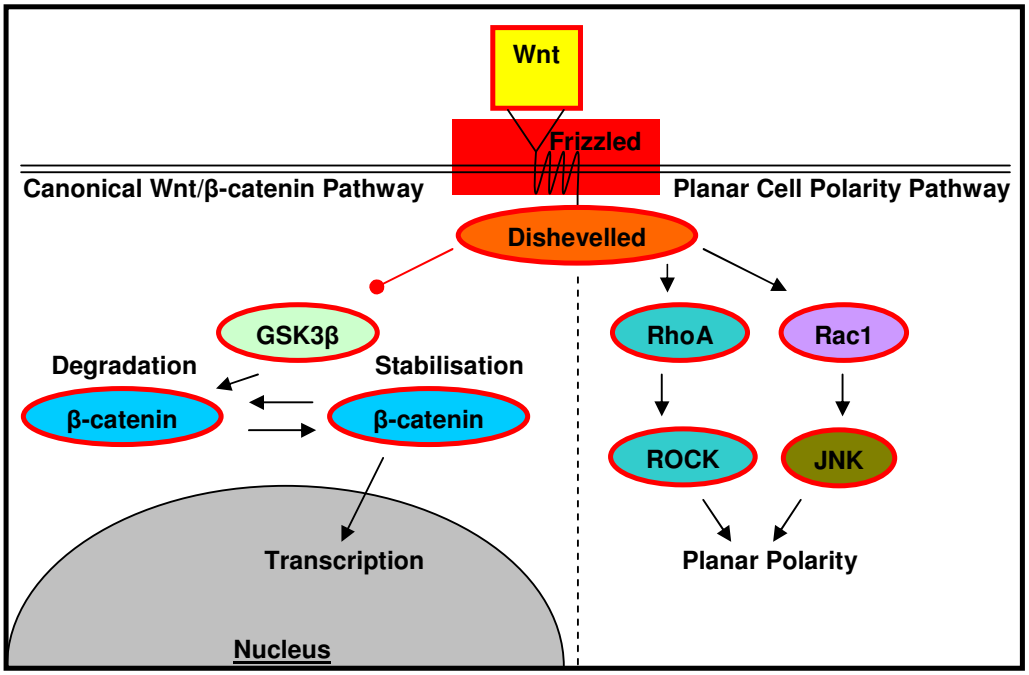


Diagram1.2 Canonical and Non-Canonical Wnt-signalling.

The diagram is based on references cited in the text.

1.4.5 Non-canonical Wnt signalling: the Planar Cell Polarity and the Calcium-pathways in the kidney

The non-canonical pathways have two major branches: the planar cell polarity pathway and the calcium pathway, which are both activated by Wnt binding to Frizzled, discussed by Habas, David and He as well as by Slusarski and Pelegri (Habas, Dawid, and He 2003; Slusarski and Pelegri 2007). The calcium pathway acts via phospholipase C (PLC) which promotes the conversion of phosphatidylinositol-4,5-biphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol which stimulate calcium release and activation of PKC, respectively, reviewed by Slusarski and Pelegri (Slusarski and Pelegri 2007). The release of calcium results in the activation of calcium-dependent proteins such as calcium/calmodulin-dependent kinase II (CamKII) and the activation of nuclear factors of activated T cells (NFATs) (Slusarski and Pelegri 2007). Both calcium as well as NFATs are capable of

inhibiting the canonical Wnt-signalling pathway (Slusarski and Pelegri 2007). The importance of NFATs in the kidneys is currently being investigated in the kidney, as so far very little is known (Burn, S. personal communication). As mentioned earlier, the planar cell polarity is perhaps more relevant for this work. The planar cell polarity is a different type of polarity compared to the apicobasal polarity. Whereas apicobasal polarity refers to the polarity across an individual cell, planar polarity is the polarity that is found along the axis of the epithelium (Davies and Garrod 1997). The majority of molecular components in the planar cell polarity pathways were identified in Drosophila, however a recent review by Wang and Nathans gives an excellent summary of the vertebrate system (Wang and Nathans 2007). The planar cell polarity pathway is not exclusively activated by the Wnt/Frizzled pathway but can also be regulated via a set of protocadherins as is discussed below. The Wnt/Frizzled planar cell polarity pathway has been shown to work in Human Embryonic Kidney 293 (HEK293) cells via the independent activation of both the RhoA and Rac1 through Dishevelled, where Rac1 activates Jun N-terminal Kinase (JNK) (Habas, Dawid, and He 2003). In vivo experiments have demonstrated that the same mechanism is important for Xenopus gastrulation and convergent extension (Habas, Dawid, and He 2003).

Evidence is emerging that both of the planar cell polarity pathways affect kidney development. Using a kidney progenitor cell culture system, Osafune and colleagues demonstrated that inhibition of either JNK1, JNK2 or Rac1 resulted in smaller cultures when inhibited in either whole kidney cultures (JNK1 and JNK2 only) or nephron progenitor cell cultures (JNK1, JNK2 and Rac1) (Osafune et al 2006). On the contrary, activation of Rac1 resulted in large cultures; the same was seen by inhibition of Rho-kinase or RhoA (Osafune et al 2006). Interestingly, inhibition of JNK1 or JNK2 resulted in a reduction in the number of progenitor cell cultures that expressed markers indicative of fully epithelialised cells which suggested that JNK1 and JNK2 might be important for the transition from a mesenchymal to epithelial state (Osafune et al 2006). Inhibition of JNK1 or JNK2 did not however disturb ureteric bud morphology, and the kidneys displayed size-wise proportionally normal numbers of branches (Osafune et al 2006). The authors concluded that a planar cell

polarity pathway is used in the kidney to regulate kidney and nephron size, however, the authors unfortunately did not look at effects on nephron size in whole cultures nor did they determine whether the planar cell polarity pathway affected proliferation rates. This is an important point, as the differences in culture sizes could well be a result of cell size rather than cell number.

The protocadherin planar cell polarity pathway, or the Dachsous system, has also been observed during kidney development. This pathway functions via the protocadherins Fat and Dachsous in addition to a protein Four-jointed, where Dachsous is antagonist of Fat, which in turn is an inhibitor of Four-jointed transcription (Casal, Lawrence, and Struhl 2006; Wang and Nathans 2007; Saburi et al 2008). The protocadherin planar cell polarity pathway appears to function independently of the Wnt/Frizzled pathway and its polarity is set up based on cellular variations in the concentration of Dachsous and Fat (Casal, Lawrence, and Struhl 2006). This planar cell polarity pathway has in fact been demonstrated to be an important regulator for collecting duct and nephron morphogenesis (Saburi et al 2008). Mutants of *Fat4* display shortened and bloated collecting duct systems as well as dilated tubules within the nephron (Saburi et al 2008). Combinations of *Fat4* mutants and mutants of either *van Gogh-like 2 (Vangl2)* or *Four-jointed* results in more severe but consistent phenotypes (Saburi et al 2008). The formation of dilated cystic tubules appears to be a result of a disruption in the mechanism that regulates the orientation of cell division (Saburi et al 2008). Normally, in the kidney, the majority of cell division are in the plane of the epithelium (Fischer et al 2006). This orientation was shown to be disrupted in mice displaying polycystic kidney disease (Fischer et al 2006) as well as in the *Fat4* mutants (Saburi et al 2008). Significantly, the distorted tubule morphology and cystogenesis did not lead to aberrant cellular differentiation, suggesting a structural organisational defect rather than a genetic one (Saburi et al 2008).

To gain a better understanding of how all types of Wnt signalling might regulate nephron induction, it is important to formally show that the β-catenin pathway is activated by Wnt signalling. Although, both β-catenin-dependent signalling and

planar cell polarity pathways have been shown in the kidney, it is still necessary to determine which Wnt signalling molecules activate these cascades since other signalling molecules such as R-spondins are also capable of binding and activating Frizzled, reviewed by Clevers (Clevers 2006).

1.4.6 Stage 2: Pretubular Aggregates towards Renal Vesicles

The pretubular aggregates form as a result of nephron induction (Saxen 1987). The pretubular aggregates are small cellular clusters of the cells from the cap mesenchyme which have received an inductive signal and are progressing towards a nephrogenic fate. A key marker for the pretubular aggregate stage is *Wnt4* expression. Wnt4 has long been known to be a crucial regulator of nephron development and *Wnt4* mutants arrest their nephrogenic development at around the stage of pretubular aggregate formation, which correlates well with the onset of *Wnt4* expression in the nephron (Stark et al 1994). The formation of the pretubular aggregates can be seen as the first structurally defined population of cells that will contribute to the nephrons; however, it is not known how these cells are selected and how they segregate out from the other cells in the cap mesenchyme which also receive the inductive signals from the ureteric bud. Recently, large scale microarray experiments have been carried out in order to characterise the genes that are important during the transition from pretubular aggregate stage to renal vesicle stage (Valerius and McMahon 2008). In the analysis by Valerius and McMahon, the authors performed microarray analyses of whole kidneys from E14.5 embryos of wild type and *Wnt4* mutant genotypes (Valerius and McMahon 2008). Unfortunately, as the microarray experiments were carried out on E14.5 kidneys, the wild type samples contained nephrons far beyond the renal vesicle stage, thus limiting the analysis somewhat. Why *Wnt4* mutants arrest at the pretubular aggregate stage is not known, and it has been suggested that *Wnt4* might not be as essential to renal vesicle formation as others have reported since a few nephrons are sometimes detected in *Wnt4* mutant mice (Kobayashi et al 2005). Other genes that are necessary for the progression towards the renal vesicle stage are genes coding for structural

components such as *α6β1 integrin* and *α-laminin* which both have been shown to be important for nephron formation (Klein et al 1988b; Sorokin et al 1990). α6β1 integrin and α-laminin protein are to a large extent similar in their pattern where both are detected at a stage preceding the epithelialisation of the mesenchymal cells, although whereas α-laminin is exclusively detected on the basal surfaces of epithelialising cells, α6-integrin is also found on the lateral and apical surfaces (Sorokin et al 1990). Inhibition of α-laminin and/or α6-integrin results in the arrest of nephron development at a pretubular aggregates stage and no polarised cells are formed (Klein et al 1988b; Sorokin et al 1990). These are interesting results because, rather than simply showing the necessity for a particular gene during nephron development, these studies determined the need for proteins that are important for structural processes such as adhesion, cell-matrix binding and apicobasal polarity formation during renal vesicle formation.

The renal vesicle represents the first epithelial structure of the nephrons. The formation of the renal vesicle from the pretubular aggregates requires the transition from a mesenchymal cell type into an epithelial type. To comprehend the mesenchymal-to-epithelial transition during nephrogenesis it is necessary to appreciate some of the changes that the cells are required to undergo during this transition (Davies and Garrod 1997). In an attempt to define what makes a cell epithelial, Davies and Garrod came to the conclusion that no specific gene or protein can fully define a single cell as being either epithelial or mesenchymal (Davies and Garrod 1997). However, the properties that a set of cells possess as a whole can generally be defined as epithelial or mesenchymal (Davies and Garrod 1997). The renal vesicle fulfils many of the criteria for being epithelial although a very immature epithelium. Of major interest and importance to the nephron is the development of three cellular polarities; the apical, basal and lateral. It is not known in what order these polarities develop in the nephron, although α-laminin and NCAM demarcates the basal surfaces and the lateral sides from a very early stage, respectively (Klein et al 1988a; Klein et al 1988b). NCAM is found at high amounts in the renal vesicle and in the pretubular aggregates, but it is also found in the condensed mesenchyme at levels that are relatively uniform to those in the nephron structures (Klein et al

1988a). Knockouts of *NCAM* are viable and, though they do have non-kidney phenotypes, no effects are reported in the kidney (Cremer et al 1994). According to electron microscopy studies carried out on rabbit renal vesicles, tight-junctions are present from an early stage but are found along the whole of the lateral surface and it is only at later time-points that the localisation is restricted towards the apical portion of the lateral surface (Minuth, Schiller, and Taugner 1981). In mature epithelia, tight-junctions are otherwise a good marker for the apical surface (Davies and Garrod 1997). In terms of cadherins, both E-cadherin and cadherin-6 are detected in the renal vesicle (Cho et al 1998), however, the disruption of *cadherin-6* results in no major phenotypes except that *cadherin-6* mutant mice show a slight delay in the mesenchymal-to-epithelial conversion and some problems with fusing to the ureteric bud (Mah et al 2000). The apical surface of the renal vesicle faces the inside, or luminal surface of the cyst and proteins that displays an apically restricted localisation in the renal vesicle, such as gp330/α2-macroglobulin receptor associated protein (Abbate et al 1994), are rare. Even the gp330/α2-macroglobulin receptor associated protein is not an ideal marker as it is also found within the cytoplasm and in cytoplasmic vacuoles (Abbate et al 1994). Desmosome components, desmoplakin 1 and 2, have been shown to present from a very early stage (Garrod and Fleming 1990). In the renal vesicle, they display a stronger localisation towards the apical surface, although they are still present basally (Garrod and Fleming 1990). The progression of the nephrons from a mesenchymal to epithelial cell state is gradual as has been demonstrated using several epithelial markers (Davies and Garrod 1995). It is only to be expected that the acquisition of a properly defined apicobasal axis will also be occurring gradually as the cells gain more functions and a greater need for a developed polarity. The most developed polarity of the renal vesicle is the basal polarity where α-laminin is rapidly deposited during epithelialisation (Klein et al 1988b).

In order to provide a morphological basis for the introduction to the nephron development around and beyond the renal vesicle stage, a series of images show the development that a nephron goes through (Fig.1.1). The greyscale images were obtained from Potter's excellent work *Normal and Abnormal Development of the*

Kidney (Potter 1972), p.46-47, and show the early human nephrogenesis from renal vesicle stage, through comma-shape body stage and until s-shape body stage. Images 1-8 are in the order of development and the stage is specified for each image. These images highlight the important structural changes that occur.

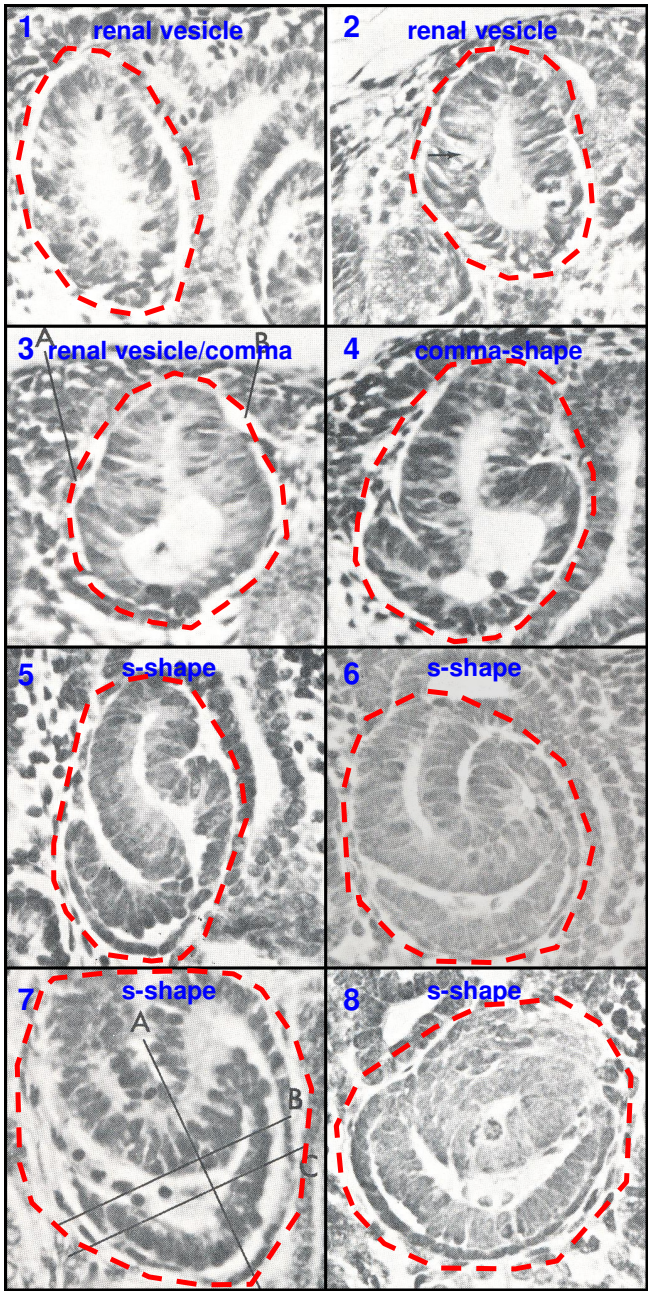


Fig.1.1 Human Renal Vesicle to S-shaped Body stage.

Original greyscale images were obtained and adapted entirely from p.46-47 (Potter 1972). Nephron stages are in order and are specified in blue text. The nephron borders are marked with red dashed lines. Letters 'A', 'B', and 'C' should be ignored.

The second polarity of the renal vesicle is a planar polarity. It is interesting to note that whereas the renal vesicle is immature in terms of its apicobasal axis, the planar polarity is already quite defined although not fully developed. The planar polarity of the renal vesicle is the same as the proximal-distal polarity of the nephron. The proximal-distal axis of the nephron is based on the terms used in the mature nephron where the nephron segments are either near or far from to the glomerulus. In the renal vesicle this nomenclature is confusing because the glomerulus has not yet developed and it is likely that the most important signalling centre for the nephron is the ureteric bud since the renal vesicle's proximal-distal axis is always oriented so that the distal nephron portion is nearest to the ureteric bud. Interestingly, nephrons can still initiate proximal-distal axis formation if induced by an ectopic source and without the orienting guidance of the ureteric bud (Davies and Garrod 1995; Kispert, Vainio, and McMahon 1998). In the mature nephron at least 8 molecularly defined regions are detectable between the glomerulus and the collecting duct (Raciti et al 2008). At renal vesicle stage, at least two genetically distinct domains exist; the distal domain and the proximal domain which contains glomerular progenitors.

Numerous proteins display entirely restricted or elevated detection in the distal domain, for example, E-cadherin (Cho et al 1998), LIM homeobox 1 (Lim1/Lhx1) (Kobayashi et al 2005; Cheng et al 2007) and Brn1/ POU transcription factor class 3 (Brn1/Pou3f3) (Nakai et al 2003), and several genes have been shown to be expressed mainly in the distal regions, for example, *Bone morphogenetic protein 2 (BMP2)*, *Dickkopf homologue 1 (Dkk1)*, *Gene regulated by oestrogen in breast cancer protein 1 (Greb1)* and *Jagged 1 (Jag1)* (gudmap.org). A smaller number of proteins have so far been shown to be primarily localised to proximal regions although WT1 (Huber et al 2000)(gudmap.org) and cadherin-6 (Cho et al 1998) are excellent examples. It is possible that the cells within the pretubular aggregates already possess some form of polarised pattern and that this only becomes sufficiently clear in the renal vesicles when the cells are sufficiently structured. The orientation of the proximal-distal axis is likely be a function of the ureteric bud as the nephrons align in a stereotyped fashion towards the bud. Knockouts of *Six2* display ectopic nephron formation on the renal capsule facing side of the ureteric buds and in

spite of this the majority of ectopic nephrons display normal proximal-distal axis alignment towards the ureteric bud (Self et al 2006), reviewed by Kopan, Cheng and Surendran (Kopan, Cheng, and Surendran 2007). The *Six2* knockout nephrons do however sporadically show a proximal-distal axis confusion, where segment specific markers display expanded regions of overlap (Self et al 2006). This does suggest that the nephrons themselves also possess a mechanism to arrange their orientation, although it could just be a mechanism that responds to cues from the ureteric bud. The nephrons are however capable of producing normal proximal-distal axes entirely in the absence of the ureteric bud. As previously mentioned, the expression of Wnts from NIH3T3 cells can induce the metanephric mesenchyme to generate typically patterned nephrons (Kispert, Vainio, and McMahon 1998). This suggests that the nephrons are capable of organising their proximal-distal axes, although the ureteric bud can probably influence the direction of the axis.

1.4.7 Stage 3: Comma-Shaped and S-Shaped Nephrons

In the progression from renal vesicles to comma-shaped bodies, nephrons are dependent on different genes to those in earlier stages. Transcription factors Lim1 (Kobayashi et al 2005) and WT1 (Armstrong et al 1993), extracellular signalling protein Wnt4 (Stark et al 1994; Kobayashi et al 2005) and growth factor FGF8 (Grieshammer et al 2005) are all likely to be important at this stage. Conditional *Lim1* knockout mice with a deletion of *Lim1* in the metanephric mesenchyme have been shown to allow nephron development to progress until the renal vesicle stage but no further (Kobayashi et al 2005). *Lim1* is expressed in the whole of the pretubular aggregates and renal vesicles and show restricted expression in the comma- and s-shaped bodies and in the immature podocytes (Kobayashi et al 2005). Interestingly, in chimaeras of *Lim1* knockout cells and wild type cells the $Lim^{-/-}$ cells are capable of contributing to the entire pretubular aggregates but not to the distal domains of the renal vesicles, nor the maturing podocytes (Kobayashi et al 2005). $Lim1^{-/-}$ cells were gradually restricted until they eventually only remained within the parietal epithelium (Kobayashi et al 2005). The exclusion of $Lim1^{-/-}$ cells from the

distal portion of the renal vesicle corresponds well with the findings that this is the nephron stage where *Lim1* is initially required since the conditional *Lim1*$^{-/-}$ mutants arrested their nephrogenic development at this stage. Another gene that has been shown to be essential for renal vesicle development is *fgf8*, which is expressed in the pretubular aggregates and in the distal portion of the renal vesicles (Grieshammer et al 2005). In the s-shaped body, *fgf8* expression is detected in the entire tubular region of the nephron but is not expressed in the parietal or visceral epithelia (Grieshammer et al 2005). Conditional *fgf8* mutant mice with no *fgf8* expression in the metanephric mesenchyme do not express *Wnt4* or *Lim1* and do not progress beyond the renal vesicle stage (Grieshammer et al 2005). Of interest is that these nephrons progress until renal vesicle stage despite not expressing *Wnt4*, which previously has been considered essential for the mesenchymal-to-epithelial transition of the nephrons. The authors speculate that perhaps FGF8 is required to maintain *Wnt4* expression and the initial *Wnt4* expression comes on at a very low level that only permits the transition to take place but subsequent development is halted (Grieshammer et al 2005). *Fgf8* was also shown to be important for the promotion of cell survival in the progenitor cell population (Grieshammer et al 2005). Conditional *WT1* mutations with a deletion of *WT1* expression in the metanephric mesenchyme have also been made (Hohenstein, P. Unpublished). Interestingly, the deletion of *WT1* at an early stage of kidney development, using *Nestin-Cre*, results in the disruption of nephron formation (Hohenstein, P. personal communication) a result previously shown using siRNA targeting of *WT1* in kidney cultures (Davies et al 2004). The deletion of *WT1* using *Pax8-Cre*, expressed in the condensed mesenchyme, results in malformed nephrons with no glomerulus, however, the nephrons are tubular and maintain their ability to correctly fuse with the ureteric bud (Hohenstein, P. Personal Communication). The transition between the renal vesicle and the comma-shape body stage is beginning to become better characterised. The use of microarray analyses to compare mice with normal kidneys and mice with mutations that halt nephron development at particular stage, e.g. *Lim1*, are yielding interesting data (Potter et al 2007). The genes mentioned above highlight some of the components which now have to be connected to actual processes and pathways during nephron formation.

The formation of the comma-shaped body is characterised by the dramatic morphological change that encompasses the development of the glomerular cleft (Fig.1.1(3,4)). The formation of the glomerular cleft is rapid and it occurs by a portion of the epithelium forming a sickle-shaped invagination through the proximal part of the renal vesicle. Some genes have been demonstrated to be necessary for proximal nephron development. Conditional knockouts of *Notch2* in the metanephric mesenchyme resulted in nephrons forming without any proximal portions, such as the proximal convoluted tubule or the glomerulus (Cheng et al 2007). The distal tubules that formed displayed some normality as they retained their capability to fuse with the ureteric bud (Cheng et al 2007). *Notch2* expression was shown to be needed for normal proliferation rates in proximal portions of the nephron and interestingly, over expression of *Notch1* resulted in nephrons that were composed entirely of proximal-tubule like segments that lacked podocyte differentiation (Cheng et al 2007). The authors reported that proximal distal polarity appeared to develop normally in renal vesicles, as shown with distally restricted detection of Lim1 and high levels of Pax2, however proximal marker cadherin-6 was never detected (Cheng et al 2007). This suggests that *Notch2* expression might be needed around the time when the glomerular cleft forms. Perhaps it is misleading to consider that the proximal-distal polarity is one polarity. Rather, it might represent two domains that display differentiation down diverging pathways and that the differentiation of one domain might not necessarily be dependent on the other. In fact the apparently normal development of distal tubules in the *Notch2* mutant nephrons support that one portion can develop normally without the other doing so. However, the continuous expression of *Notch1* which rescued the *Notch2* mutant and induced proximal tubule development also appeared to induce the entire nephron to become proximal and this inhibited podocyte differentiation (Cheng et al 2007). This could indicate that podocyte differentiation is in fact dependent on normal proximal or distal tubule development but equally, it could a direct result of *Notch1* expression. It is important to bear in mind that the proximal and distal domains of the renal vesicle do not necessarily correspond to the proximal and distal segments of the mature nephron. The distal domain of the renal vesicle might correspond to the entire tubular section of the mature nephron and the proximal part, constitute only the glomerulus. To

elucidate the fate of cells in the renal vesicle, lineage maps are required. The discussion regarding *Notch2* was to point out that at least one gene has been shown to be important for proximal nephron development around the time of glomerular cleft formation. It has not been shown that *Notch2* expression is necessary for the formation of the glomerular cleft; in fact, no genes have to my knowledge been directly linked to glomerular cleft formation.

The importance of understanding glomerular cleft formation lies in the structure that it gives rise to. Glomerular cleft formation is accompanied by the formation and differentiation of the parietal and visceral epithelia which gives rise to the cells in the Bowman's capsule and the podocytes, respectively (Saxen 1987). Without these structures, no glomerulus will form and the nephron will not be functional. The structures in the s-shaped body have been highlighted in (Fig.1.2) and its development is shown in (Fig.1.1).

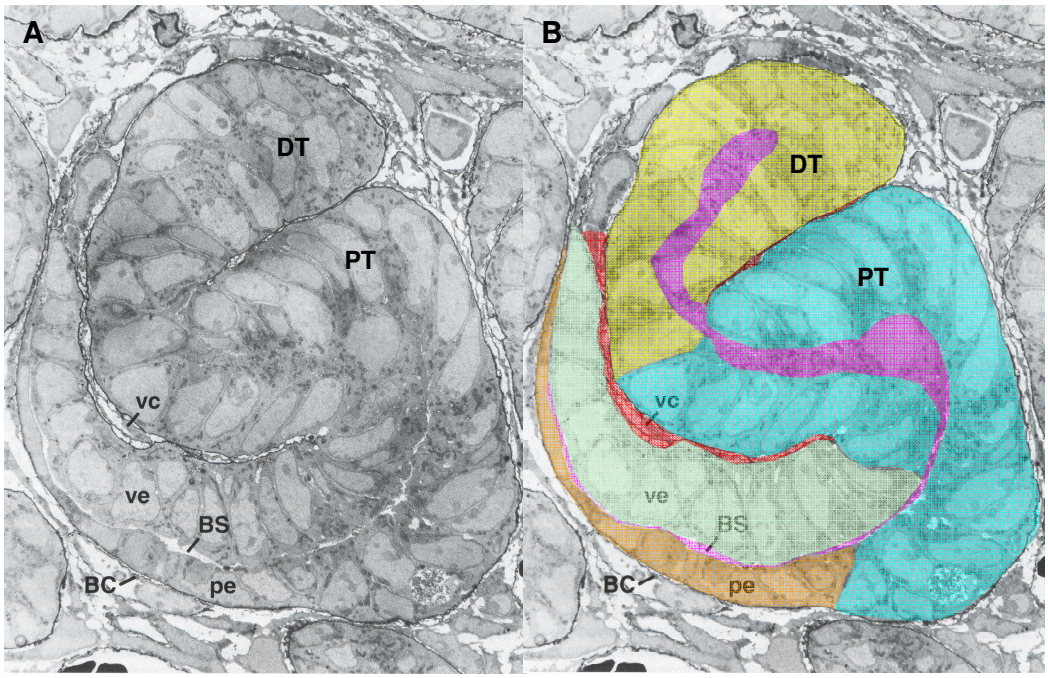


Fig.1.2 Mouse S-Shaped body nephron with labelled structures.

This electron micrograph was obtained and adapted from p.232 (Vize, Woolf, and Bard 2003). (A) and (B) shows the same nephron in greyscale. (B) Shows a superimposed coloured overlay to demark important structures. The original image annotation describes vc-vascular cleft, ve-visceral epithelium, pe-parietal epithelium, BS-Bowman's space, BC-Bowman's capsule. Additional annotations: DT-distal tubule, PT-proximal tubule.

Several hypotheses could be presented to describe glomerular cleft formation. Saxen suggested a model by cell detachment (Saxen 1987), however, invagination as in Drosophila ventral furrow formation or during sea urchin gastrulation, might also provide a realistic explanation. From observing the detailed images of human nephron development by Potter, (Potter 1972) (Fig.1.1) it is relatively clear that the cells undergo major morphological changes; in particular those cells producing the parietal epithelium.

The s-shaped body contains the majority of building blocks required for the subsequent maturation of the nephron into the mature nephron form. The subsequent development of the nephron is mainly characterised by its elongation and the concurrent segmentation and differentiation. As the nephron elongates it acquires the expression of a large number of genes such as the members of the solute carrier family which often display segment-specific gene expression (Raciti et al 2008).

1.5 Rac1 and Rho-kinase

The paragraphs above introduce the development of the kidney in order to provide a background to better understand the analyses carried out to elucidate the roles that the Rho-GTPases play during kidney morphogenesis. Briefly, the reason for investigating the role of the Rho-GTPases during kidney development is because they have been strongly implicated, but not yet shown, to act during this process. This makes them an ideal target to both better our understanding of kidney organogenesis but also to produce new and exciting data. The following sections, aim at providing a general introduction to the Rho-GTPases although each protein that has been investigated, is dealt with more thoroughly in their respective results chapters.

1.5.1 Introducing the Rho-GTPases

The Rho-GTPases belong to the Ras superfamily of proteins which comprises the Ras, Rho, Rab, Arf, Ran, Rheb, Rin/Rit and Rad/Gem subgroups, as reviewed by Aznar and Lacal (Aznar and Lacal 2001) and Zohn (Zohn et al 1998). The Rho-GTPase subgroup consists of at least 14 genes of which the most notable are *Rac1*, *Rac2*, *RhoA* and *Cdc42*, these are presented in full in Table 1.2 (Aznar and Lacal 2001). Characteristically for the Rho-GTPases is the ability to cycle between being bound to guanine diphosphate (GDP) and guanine triphosphate (GTP), which are their inactive and active states, respectively (Shih et al 1986).

Gene	**Mus musculus aliases**	**Homo sapiens aliases**
RhoA	*Arha, Arha1, Arha2*	
RhoB	*Arh6, Arhb,*	
RhoC	*Arh9, Arhc*	
Cdc42	*CDC42Hs*	*G25K*
TC10	*Rhoq, Arhq, TC10A*	
Rac1	-	
Rac2	-	
Rac3	*Rac1B*	
Rho6/Rnd1		
Rho7/Rnd2	*RhoN*	*ARHN, RHO7, RhoN*
Rho8/Rnd3/RhoE	*Arhe, RhoE*	
RhoD	*Arhd, Rho, RhoHP1, RhoM*	
RhoH	*Arhh*	*TTF*
RhoG	*Arhg, Sid10750*	

Table 1.2 The Rho-GTPase family

The genes were compiled from (Zohn et al 1998; Aznar and Lacal 2001). Gene name aliases were determined using Entrez Gene (Maglott et al 2007).

The cycling of Rho-GTPases between their active and inactive state is regulated by three major classes of proteins, the guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). GEFs, increase the exchange of GDP for GTP, GAPs enhance hydrolysis of GTP to GDP and GDIs inhibit the exchange of and/or the hydrolysis of GTP, as

reviewed by Van Aelst and D'Souza-Schorey (Van Aelst and D'Souza-Schorey 1997). The regulation of GTPase activity is made additionally complex by several processes such as spatiotemporal regulation, multi-protein complexes as well as by the sheer number of regulators (Kwan and Kirschner 2005). In a review by Buchsbaum, the author states that an excess of 60 *GEFs* have been identified in the mammalian genome (Buchsbaum 2007).

The GTPase family also have the capacity to regulate each other's activities. It has been shown that Cdc42-GTPase activation can result in the activation of Rac1 and subsequently RhoA in National Institute of Health 3T3 (NIH3T3) fibroblast cells (Nobes and Hall 1995) but evidence presented by (Sander et al 1999) provided evidence that active Rac1, induced by platelet-derived growth factor (PDGF), is also capable of down-regulating RhoA activity. (Rosenfeldt et al 2006) also found negative regulation exerted by Rac1 on Rho. They showed that in constitutively active *Rac1* mutant cells, stress-fibre formation, which is normally produced via Rho activation, was in fact inhibited. The cells that expressed the mutant *Rac1*, proved to be unable to bind p21-activated kinase 1 (PAK1), and through this lost their ability to inhibit Rho activation. PAK1 was shown to bind Rho GEF, P115RhoGEF which if inhibited results in a loss of Rho-induced stress-fibre formation (Rosenfeldt et al 2006). The introduction of dominant negative *PAK1* resulted in the retardation of the ability of Rac to inhibit thrombin-induced stress fibres (Rosenfeldt et al 2006). These results suggest an direct relationship between Rac and Rho via PAK1 (Rosenfeldt et al 2006). This is interesting, particularly in light of that the Wnt/Frizzled planar cell polarity pathway activates both Rac1 and RhoA in mammalian cells (Habas, Dawid, and He 2003).

The examples described above emphasise the versatility in regulation of activation and inactivation which is also reflected by the large number of processes where GTPases are involved. Cell growth, proliferation, cell cycling, transformation, migration and polarity are some of the functions so far found to be controlled by Rho-GTPases (Ridley et al 1992; Nobes and Hall 1995; Gu et al 2003; Benitah et al 2005) and extensively reviewed by Ridley (Ridley 2001).

Rac1 has long been known to regulate actin polymerisation via the 'WASP family Verproline homologous' (WAVE) proteins which activates the Actin related protein 2/3 (Arp2/3) complex as shown and reviewed by Ridley, Eden and colleagues (Ridley et al 1992; Eden et al 2002) and Bompard and Caron (Bompard and Caron 2005). Rac1 has also been shown to mediate cell-cell adhesion by stabilising cadherin-catenins complexes, reviewed by Fukata and colleagues (Fukata et al 1999). RhoA is a key regulator for the formation of actin-myosin contractile stress fibres as well as for formation of focal adhesion complexes (Ridley and Hall 1992; Fukata et al 1999). The close connections which GTPases have to the regulation of cellular adhesion and the cytoskeleton, as well as to gene regulation, links them to mechanisms that may be fundamental to the morphogenesis of a tissue where cell migration, adhesion, cytoskeletal remodelling and cellular differentiation may be means of normal morphogenesis.

1.6 Aims of thesis

The aim of this thesis is to improve the current understanding of mechanisms involved in ureteric bud branching morphogenesis and nephron formation. The focus has been on understanding what roles Rac1-GTPase and Rho-kinase might play during these processes. This is important because these proteins have been implicated, either directly or indirectly, in both nephrogenesis and in ureteric bud branching. Rac1 and Rho-kinase are both central proteins in the Wnt/Frizzled planar cell polarity pathways, cellular migration regulation and organising actin and actin-myosin structures. These are mechanisms that might hold a significantly place in kidney development.

1.7 Main findings of the thesis

The significant data described herein show that Rac1 regulates the size and branching of kidneys in culture via cell proliferation but Rac1 does not control

nephron formation or maturation, nor ureteric bud differentiation. Rho-kinase is an important protein during nephron formation, nephron apicobasal polarity formation, glomerular cleft formation and for proximal-distal nephron patterning. Rho-kinase regulates cell size in the ureteric bud. Rho-kinase is likely to act via actin-myosin contraction.

Chapter 2

Materials and Methods

2.1 Dissections and organ culture

2.1.1 Mouse strains

Pregnant CD1 mice were obtained from BRR-BRF-Phase II Little France, Edinburgh EH16 4SB. Mice were sacrificed by cervical dislocation. The lower abdomen was cut and the uterus removed.

2.1.2 Dissection of E11.5 kidneys

Using forceps and handheld surgical blades E11.5 CD1 uteri were dissected in Minimum Essential Medium Eagle's (M5650/SIGMA) in 90mm petri dishes (633185/Greiner Bio-One). Embryos, within their amniotic sacs, were removed. The embryos were immediately beheaded using 25gauge needles (300600/BD Microlance 3) attached to 1mL syringes (300013/BD Plasipak). This type of needle setup was used for all dissections on the embryos. All further directions will described with regards to one embryo and the same procedures were carried out on all embryos. Two incisions were carried out on the embryo to isolate the region where the kidneys are located. The first incision was made rostral to the developing hind legs and the second incision was made caudal to the developing hind legs. To expose the kidneys for dissection, the embryo was placed with its ventral side facing down and one needle was used to penetrate the embryo from the dorsal side through the developing spinal cord all the way through. In this manner the embryo was pinned to the petri dish. A second needle was used to cut through the embryo in a dorsal to ventral motion and sequentially in a rostral to caudal direction to make a cut through the most medial region. The left hand and right hand sides were separated and placed with their lateral surfaces facing down. At this stage the embryonic kidneys were fully visible and were carefully dissected out from the rest of the embryo. Using a glass pipette the kidneys were transferred to complete kidney culture medium (CKCM) (Minimum Essential Medium Eagle's with 10% calf serum

(Biosera), 100units Penicillin + 100µg/mL Streptomycin (P4333/SIGMA) and placed at 37°C in the culture hood.

2.1.3 Dissection of E11.5 spinal cord

The section of the embryo caudal to the brainstem and rostral to the forelimbs was used for this dissection. The same setup was used to dissect the spinal cord as was used for the kidneys. The regions lateral to the spinal cord were removed to leave the spinal cord. A cut was made through the most dorsal part of the spinal cord in a rostral to ventral direction. The ventral part of the spinal cord was not cut through. The spinal cord was unfolded outwards with the most dorsal parts being on the opposite sides of the roughly rectangular tissue and the ventral parts being in the centre. Cuts were made halfway between the most dorsal part and the most ventral part of the spinal cord on each side to produce two strips of dorsal spinal cord. The ventral parts were discarded. All dorsal ganglion remnants were removed and the remaining tissue was cut into small rectangular pieces.

2.1.4 Separation of ureteric bud and metanephric mesenchyme

Dissected kidneys were transferred from complete culture medium to Minimum Essential Medium Eagle's containing Trypsin EDTA (T4174/SIGMA) to a final concentration of 1x (1/10 dilution of 5g/L porcine trypsin and 2g/L EDTA 4Na in 0.9% NaCl as specified by SIGMA). The kidneys were incubate for 10min at 37°C for dissections aimed at ureteric bud isolation or 5min at 37°C for dissections aimed at metanephric mesenchyme isolation. Following incubation, rudiments were transferred to CKCM where the metanephric mesenchyme was separated from the ureteric bud using the above described needle setup.

2.1.5 Surface-medium interface kidney cultures

Trowell grids were placed in 35mm petri dishes (627160/Greiner Cell Star). Isopore membrane filters (TMTP02500/MILLIPORE) cut to appropriate sizes ca 1 cm2 were placed upon the Trowell grid. Sufficient CKCM was added to the petri dish in order for the medium to reach the membrane filter without submerging it. Dissected kidneys were placed on the membrane filters and the whole culture dish placed in the incubator at 37°C with 5% CO_2.

2.1.6 Metanephric mesenchyme induction with spinal cord

Metanephric mesenchyme was dissected from the ureteric bud as described above. Spinal cord was dissected as described above. Surface-medium interface kidney cultures were set up as described above. Spinal cord rudiments were placed on the membrane filter and the metanephric mesenchyme placed upon the spinal cord. Cultures were incubated at 37°C with 5% CO_2. Trans-filter setups were also carried out where three isopore filters were placed on top of each other with the spinal cord between the bottom two and the metanephric mesenchyme on the top filter. For these setups, the bottom two filters were removed after 24hrs of induction leaving the metanephric mesenchyme for further culturing.

2.2 Microscopy

2.2.1 Sandwich Mount for Confocal Scanning

A large rectangular coverslip 22mm x 64mm (VWR INTERNATIONAL) was placed upon a glass slide (Blue Star/CHANCE PROPPER). Two small coverslips 22mm x 22mm (No.1½/CHANCE PROPPER) were attached on opposite sides of the larger coverslip using nail varnish (PrettyinPink/LIZZIE). The fixed and stained sample was placed in the empty space between the two smaller coverslips and

immersed in a 1:1 (1xPBS:Glycerol) solution. A second large rectangular coverslip was placed on top and as such enclosing the sample. For long term storage the sides were sealed with nail varnish to prevent drying.

2.2.2 Confocal microscopy

The majority of samples scanned with a confocal microscope were scanned on a Leica TCS-NT laser scanning confocal microscope. A small portion of samples were scanned on a Zeiss LSM510 CLSM. Leica lenses used were: 5x HC PL FLUOTAR 5x/0.15; 10x HC PL FLUOTAR 10x/0.30 PH1; 20x HC PL FLUOTAR 20x/0.50 PH2; 40x PL FLUOTAR 40x/1.00 OIL PH3; PL APO 63x/1.32 OIL. Imersol fluorescent microscopy oil was used where appropriate (Imersol 518F/Zeiss).

2.3 Immunohistochemistry

2.3.1 General protocol for fluorescent immunohistochemistry in kidney cultures

(1) 10 min. fixation in −20°C methanol (M/4000/17/Fisher Scientific) at room temperature (RT.)
(2) 30 min. wash in 1x Phosphate Buffer Saline (1xPBS) (0.01M phosphate buffer, 0.0027M KCl, 0.137M NaCl, in dH_2O, pH 7.4 specified by SIGMA) (P4417/Sigma) at RT.
(3) 24 hrs. Primary antibody incubation at 4ºC.
(4) 30 min. wash in 1xPBS.
(5) 24 hrs. Secondary antibody incubation in 1xPBS at 4ºC.
(6) 30 min. wash in 1xPBS at RT.
(7) Mount as described above.

2.3.2 Antibody-specific alterations to the general protocol for immunostaining

For Phospho-Histone 3-(Ser10) (9701/CELL SIGNALLING), all steps using 1xPBS were modified so that the PBS was substituted for 1x Tris Buffered Saline (0.05M Tris and 0.15M NaCl, in dH_2O, pH 7.6 specified by SIGMA) (T5030/SIGMA). The 1x Tris Buffered Saline solution contained 5% goat serum (G9023/SIGMA). Each washing step was extended with 30min.

For staining with Dolichos biflorus Lectin-FITC conjugated (L2785/SIGMA), the lectin was diluted in 5% milk (Dried Skimmed Milk/MARVEL) in 1xPBS.

2.3.3 Primary and secondary antibodies

Mouse IgG anti-Calbindin D-28K (ab9481/ABCAM), 1:100. Rabbit IgG anti-Calbindin D-28K (ab1778/CHEMICON), 1:100. Rabbit IgG anti-Laminin (L9393/SIGMA), 1:100. Mouse IgG anti-NCAM (C9672/SIGMA), 1:100. Mouse IgM anti-CD15 (C7798/SIGMA), 1:100. Mouse IgG anti-E-cadherin (610181/BD), 1:100. Mouse IgG anti-Cytokeratin (C2562/SIGMA), 1:100. Mouse IgG anti-WT1 (sc-192 /SANTA CRUZ), 1:400. Rabbit IgG anti-Phospho-Histone 3-(Ser10) (9701/CELL SIGNALLING), 1:100. Rat IgG anti-L1 (MAB5272/Chemicon), 1:100. Mouse IgG Anti-BrdU (B2531/SIGMA), 1:50. Mouse IgG anti-ROCK-II (610623/BD Transduction Labs), 1:100. TO-PRO-3 Iodide (T3605/MOLECULAR PROBES), 1:250. Propidium Iodide (P3566/MOLECULAR PROBES), 1:1000. Phalloidin FITC-conjugated (P5282/SIGMA), 1:100. Dolichos biflorus Lectin FITC-conjugated (L2785/SIGMA), 1:100) Anti-mouse-IgG-FITC (F0257/SIGMA), 1:200. Anti-mouse-IgG-TRITC (T5393/SIGMA), 1:200. Anti-mouse-IgM-FITC (M9259/SIGMA), 1:100. Anti-rabbit-FITC (F9887/SIGMA), 1:200. Anti-rabbit-TRITC (T5268/SIGMA), 1:200. Anti-rat-IgG-Alexa Fluor 647 (A21472/MOLECULAR PROBES), 1:100. Anti-mouse-IgG-Alexa Fluor 647 (A31571/MOLECULAR PROBES), 1:100.

2.3.4 Testing of antibodies for kidney fluorescent immunohistochemistry

All new antibodies were controlled as follows: (a) Primary antibody only; (b) Secondary antibody only; (c) Primary and Secondary antibodies. Kidneys were scanned using confocal microscopy or imaged on the fluorescent microscope to test for fluorescence by the primary antibody and for non-specific binding of the secondary antibody. For some antibodies (e.g. anti-L1), additional tests had to be carried out to ensure specificity of binding.

2.4 Protein inhibitors

Protein inhibitors were added directly to the kidney cultures by adding the reconstituted inhibitor solutions to the kidney culture medium. NSC23766 (553502/CALBIOCHEM) was reconstituted in dH_2O to a stock concentration of 10mM. NSC23766 was used at a maximum concentration of 100µM. Y-27632 (Y0503/SIGMA) was reconstituted in dH_2O to a stock concentration of 5mM. Y-27632 was used at a maximum concentration of 20µM. Glycyl-H1152 dihydrochloride (2485/TOCRIS) was reconstituted in dH_2O to a stock concentration of 5mM. Glycyl-H1152 dihydrochloride was used at a maximum concentration of 2.5µM. 2,3-Butanedione monoxime (203984/CALBIOCHEM) was reconstituted in DMSO to a stock concentration of 5M. 2,3-Butanedione monoxime was used at a maximum concentration of 10mM. Methotrexate (M8407/SIGMA) was reconstituted in 1M NaOH to a stock concentration of 50mM. Methotrexate was used at a maximum concentration of 500nM.

For experiments where inhibitors were added, the control conditions, denoted as CKCM (Complete Kidney Culture Medium) or “controls/control conditions”, contained a volume of inhibitor-solvent equal to the maximum used in experimental conditions.

2.5 Proliferation assay using 5-bromo-2-deoxyuridine

2.5.1 Marking Proliferating Cells in Kidneys using BrdU

E11.5 CD1 embryos were dissected as described above and cultured as described in using surface-medium interface kidney cultures. Experimental kidneys were cultured in CKCM with 75µM NSC23766 (553502/Calbiochem). Control kidneys were cultured in CKCM without any added NSC23766. At t=30hrs 5-bromo-2-deoxyuridine (BrdU) (B9285/Sigma) was added to make a final working concentration of 100µM. Kidneys were exposed to BrdU for 16hrs.

2.5.2 Immunohistochemistry for 5-bromo-2-deoxyuridine

At t=46hrs kidneys on filters were treated according to the following protocol:

(1) 10 min. fixation in −20°C methanol (M/4000/17/Fisher Scientific) at RT.

(2) 30 min. wash in 1xPBS (P4417/Sigma, 0.01M phosphate buffer, 0.0027M KCl, 0.137M NaCl, in dH_2O, pH 7.4 specified by SIGMA) at RT.

(3) 30 min. fixation in 4% paraformaldehyde (PFA) (15,812-7/Sigma) in 1xPBS at RT.

(4) 30 min. wash in 1xPBS at RT.

(5) 7 min. wash in 0.5 mg/mL trypsin (T4174/SIGMA) at 37ºC.

(6) 1.5 hrs. fixation in 4% PFA in 1xPBS at RT.

(7) 30 min. wash in 1xPBS at RT.

(8) 60min DNA-denaturing in (95% formamide (F7503/Sigma), 5% 0.15M trisodiumcitrate (10242/BDH)) at 70ºC.

(9) 30 min. wash in 1xPBS at RT.

(10) 24 hrs. Primary antibody incubation (1:200 anti-calbindin D28k-rabbit (Ab1778/Chemicon) and 1:50 anti-BrdU-mouse (B2531/Sigma) in 1xPBS) at 4ºC.

(11) 30 min. wash in 1xPBS at RT.

(12) 24 hrs. Secondary antibody incubation (1:100 FITC-conjugated anti-rabbit IgG (F0382/Sigma) and 1:100 TRITC-conjugated anti-mouse IgG (T5393/SIGMA) in 1xPBS) at 4ºC.
(13) 30 min. wash in 1xPBS at RT.

Kidneys on filters were mounted on Sandwich Mount slides and scanned using a Leica TCS-NT confocal microscope. FITC and TRITC channels were scanned sequentially to ensure minimal levels of cross-talk. Images were captured at 3µm intervals through the whole kidneys.

2.5.3 Quantification of cell proliferation

The quantification of BrdU positive nuclei within the tips was difficult and required the strict adherence to a relatively complicated but necessary protocol. In order to make the measurements unambiguous and replicable, the protocol is described in full detail. All lines and points discussed below are shown in (Fig.2.1) p66.

For each confocal stack, every tip within each stack was analysed. All sections of each stack were viewed and ever tip was identified. For each tip, it was determined which image within the stack it was where the tip was at its widest. This was identified by eye and for each tip, this image was considered to be the "middle section" of that tip.
Each stack was opened using Scion Image Alpha 4.0.3.2 and calibrated to a scale of 1024pixels:500µm using the Set scale function. This calibration was applicable to all images as they were all captured using the 20x lens (HC PL FLUOTAR 20x/0.50 PH2/Leica) with the same Leica TCS-NT confocal microscope. All images were converted to greyscale using the Greyscale function. For area measurements, the image that had been captured using the FITC-channel, thus showing the Calbindin D-28K protein distribution, was opened and for the BrdU nuclei counts, the image captured using the TRITC-channel was opened.

The following protocol was used to (i) determine the tip boundaries, (ii) measure the tip area in μm^2 and (iii) count BrdU positive nuclei.

i) Determining tip boundaries

In order to determine whether a 'tip' should be considered as one or two tips the following screen was carried out. The middle section image showing the Calbindin D-28K protein localisation was opened and the two points furthest apart in the tip were identified and a 100µm line was drawn from each point towards the other point. For illustrative/explanatory purposes these lines were named "Line 1" and "Line 2" and they are depicted as dashed double-headed arrows and the points are termed "Widest point 1" and "Widest point 2" and are shown as blue circular marks. If the lines overlap by >25% the tip was considered to be one tip and if they overlapped by <25% the tip was separated into two tips. To determine the end of the tip and the beginning of the stalk a point was identified approximately equidistant to the two widest points that is the furthest away from the stalk. This point shall be considered as "Alternative point 1" and was here shown using a purple circular mark. A 100µm line was drawn from this point towards the stalk to a point medial to the ureteric bud edges where that point was located in the stalk region or in a region directly neighbouring the stalk (this will depend on the size of the tip). This line was named "Line 3" and it is represented by a solid double-headed arrow. The point shall be named "Alternative point 2" and it was marked with a yellow circular mark. Draw a line through alternative point 2 that was perpendicular to line 3 so that the line intercepts the ureteric bud edges. In the case where that line would not immediately connect with the ureteric bud edges the line or lines should be drawn to ureteric bud margins that are nearest to the alternative point 2. This line shall be named "Line 4"and it was shown as a dashed white line that intercepts alternative point 2. This protocol allowed for the consistent specification of the ureteric bud tip regions for every tip analysed.

ii) Measurement of the tip area in μm^2

Using the Polygon selection function the outer edges of the tip were traced and using the Measure function the surface area of the selection was measured and the area measurement was recorded. This was repeated a total of 3 times per tip to provide a mean measure and ensure increased accuracy as the area was identified by hand.

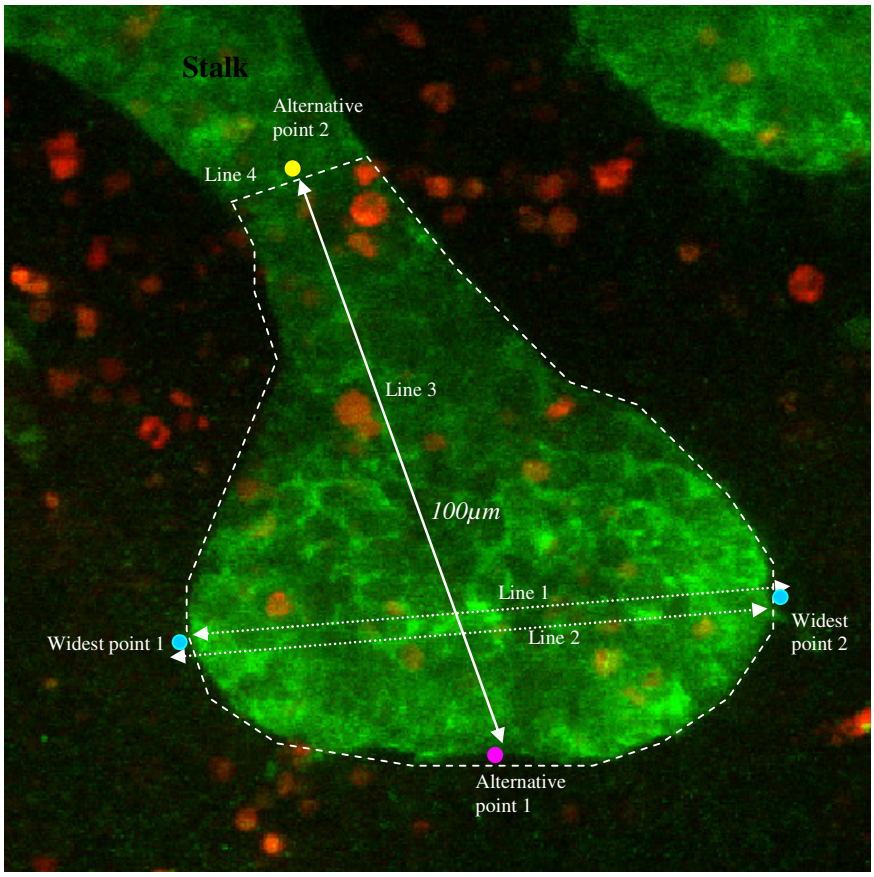
iii) Counting BrdU positive nuclei.

The image showing the BrdU (TRITC channel only) was and converted to greyscale using the Greyscale function. The Background Intensity and its Standard Deviation was measured using the Measure function and the data was recorded. The image was thresholded using the Threshold function to a level equal to ((Background Intensity x 3) + Standard deviation) as previously used by Michael and Davies (Michael and Davies 2004).

Pixels with a value above this threshold were recorded as BrdU positive. To resolve whether closely positioned pixels belonged to the same nuclei the threshold value was lowered and subsequently raised again. All positive nuclei within the tip boundary were recorded.

This protocol was repeated for the stack sections that were 3µm above and below the middle section of each tip. The alternative point 2 which indicates the tip/stalk boundary was directly determined by drawing a 100µm line from alternative point 1 which was determined by eye based on the work done on the middle section. Nuclei appearing in more than one image were counted as separate nuclei to ensure than no two or more nuclei were wrongly considered as one.

All tips within each stack were analysed and the total number of nuclei were deduced for each tip and expressed as (No. of BrdU positive nuclei per $1000\mu m^2$).



Not to scale

Fig.2.1 BrdU analysis in ureteric bud tips

2.6 Proliferation assay using phosphorylated histone 3 as a mitosis marker

2.6.1 Kidney culture conditions

E11.5 CD1 embryos were dissected and cultured as described above. Experimental kidneys were cultured in CKCM with 75µM Rac1 inhibitor NSC23766, 150nM Methotrexate (MTX) (M8407/SIGMA) or 250nM MTX. At t=48hrs kidneys were fixed in -20ºC methanol. Staining protocols were followed for Phospho-Histone 3-(Ser10). Kidneys were stained for rabbit-Phospho-Histone 3-(Ser10) (H3(p)) and mouse-E-cadherin. These were detected with anti-mouse-IgG-FITC and anti-rabbit-IgG-TRITC and counterstained with TO-PRO-3 Iodide. Kidneys were sequentially

scanned at 5µm section intervals from the top to the centre of the kidney using a 40x lens (Leica).

2.6.2 Quantification of mitotic cells

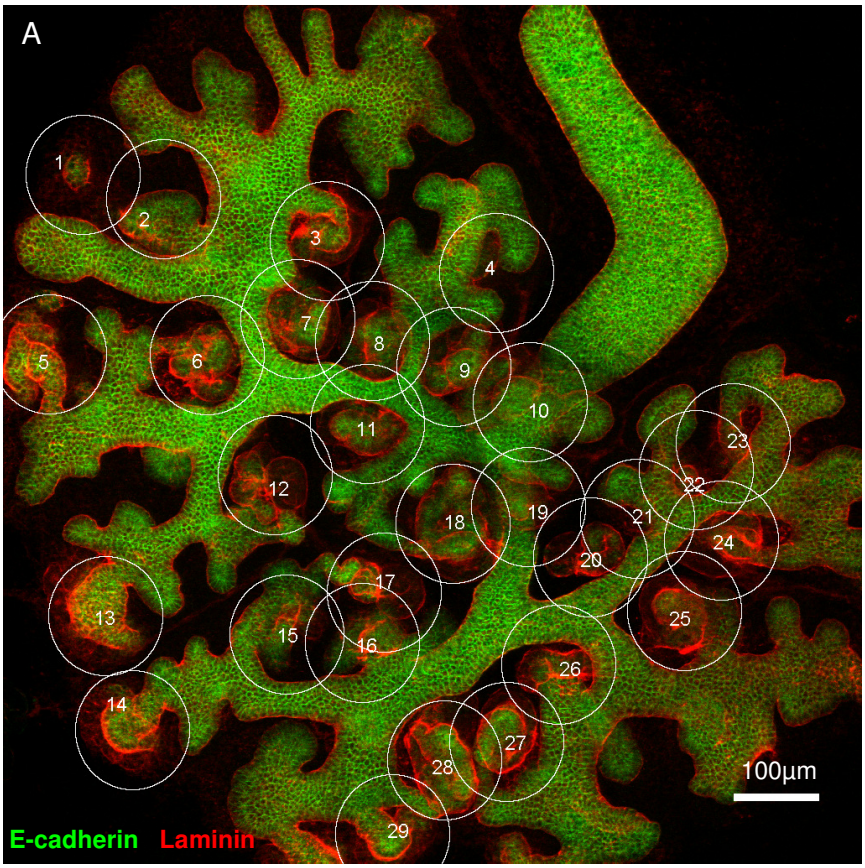
The top section of the kidney was identified as the first section displaying E-cadherin positive ureteric bud staining. The sections below this, at a depth of 5µm and 10µm were chosen for analysis. Using ImageJ 1.40g the TO-PRO-3 stain was separately and manually enhanced using the Enhance Contrast function to ensure all nuclei were clearly detectable. The image was further processed using the Smooth function and made into a binary image using the Make Binary function. In order to split nuclei that on the image appeared merged, the image was treated using the Watershed function. The number of nuclei was counted using the Analyze Particles function, set to detect particles of a size between 300 and 5000pixels (73µm2 – 1220µm2). These parameters included most whole nuclei whilst excluding background noise. The number of H3(p) positive nuclei was counted manually using the Cell Counter plug-in as this appeared to be the most reliable method. The mitotic cells were quantified as a percentage of the total number of nuclei per analysed slide.

2.7 Quantitative analysis of nephron abnormality

E11.5 CD1 embryos were dissected and cultured as described above. Controls and experimental cultures were initially kept in CKCM for 48hrs and then transferred to either CKCM or medium with 1.25µM Glycyl-H1152 dihydrochloride and cultured for an additional 72hrs. The quantification of morphological and gene expression abnormality required the following protocol.

All nephrons in samples were identified using rabbit anti-Laminin and mouse anti-E-cadherin antibodies detected with anti-laminin-IgG-TRITC and anti-mouse-IgG-FITC. Each identified nephron was individually labelled and characterised (Fig.2.2 A,B). Each nephron was given a record of nephron stage, morphology and E-

cadherin expression as described in (Fig.2.2B). Nephrons were staged using an adapted ontology from GUDMAP (www.GUDMAP.org)/ (Little et al 2007). Nephrons were staged as: Renal Vesicle (RV) – epithelial cyst with no evidence of glomerular cleft formation; Comma- and S-shaped body (CSB) – more mature cyst/tubule with glomerular cleft; S-shaped body + (SB+) – elongated tubular structure more advanced than S-shaped body stage. The CSB category was created to contain nephrogenic structures more mature than RV stage (clear glomerular cleft and/or some tubular elements) but not sufficiently tubular or elongated for SB+ stage. Nephron morphology was categorised as normal or abnormal compared to control nephrons. E-cadherin expression was categorised as normal or abnormal where E-cadherin expression showed normal segment specific expression or ectopic proximally expanded expression compared to control nephrons. In both cases, samples that could not be assigned with certainty to normal or abnormal categories were classified as "ambiguous". All image analysis was carried out using ImageJ (v.1.40g).



B Nephron annotation

- Developmental stages
  - •RV (renal vesicle stage)
  - •CSB (comma & s-shaped body stage)
  - •SB+ (post-s-shaped body stage)
- Morphology
  - •normal
  - •abnormal
  - •ambiguous
- E-cadherin expression
  - •normal
  - •abnormal
  - •ambiguous

Fig.2.2 Quantitative analysis of nephron abnormality

Fig.2.2 (A) shows a representative confocal section of a stack where nephrons have been identified and numbered – white circles and numbers – and each nephron has been classified according to its developmental stage, morphology and protein localisation; ontology shown in (B).

2.8 Segment specific gene expression analysis

This contains protocols and specific information explaining the analysis of the nephron proximal-distal axis gene expression.

2.9 Standard laboratory techniques

2.9.1 Primers design

Primers were designed using Primer3 (v. 0.4.0) (http://frodo.wi.mit.edu/) and FASTA sequences obtained from NSBI- Entrez Gene. Gene names, Gene IDs and primers are shown below.

Gene name	GI number	Amplicon	Forward primer	Reverse primer
Brn1/Pou3f3	GI:112421035	127	CAGCCTACAGCTGGAAAAGG	GGTACCCACCTGCGAGTAGA
Hunk/MAP-V/MAK-V	GI:118131024	372	TCCCATCCTTCAAATGCTTC	CATCAGTAGCCACAGGCTGA
THP/Uromodulin Uromucoid	GI:145301571	156	TCAGCCTGAAGACCTCCCTA	GAAAAGCCTCAGTGGACAGC
Hes5	GI:145966833	380	AAGAGCCTGCACCAGGACTA	AGCCTTCGGAAGAAGGTAGC
Irx3	GI:55741684		GACGAGGAAGAGAGCAAACG	TCGTCCGAGTCGCTAGTTTT
Cdh-6/K-cadherin	GI:110665731		ACATACAGGCCACCAAGAGG	CGTGACTTGGACCACAAATG
Hey1	GI:117606331		AGCAGTGAGGTGAAGGGAGA	AACGGTGAAATCCGTGAGAC
SGLT2/Slc5a2	GI:117606331		ATTGTCTCGGGCTGGTATTG	TTAGAGCAGCCCACCTCAGT
β-actin	GI:142378181		TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA

2.9.2 RNA isolation, cDNA synthesis and PCR reactions

Cultures were set up in control conditions and experimental conditions as specified in Chapter 4.4. Cultures were set up in duplicates with a total of 7-10 kidneys per condition. At the point of RNA isolation, the kidneys were removed from the filters using glass pipettes and placed in ice-cold Minimum Essential Medium Eagle's (M5650/SIGMA) in a 1.5mL Eppendorf tube (616201/Greiner Bio-One) and kept on

ice until all kidneys were collected. The kidneys were gently spun down using a micro-centrifuge (MICROCENTAUR/MSE) and the Minimum Essential Medium Eagle's was removed. The RNA isolation was carried out using SV total RNA isolation (Z3100/Promega) according to product protocols. All solutions and components described below were part of that product. Briefly, the kidneys were dissociated in 175µL RNA Lysis Buffer by vigorous pipetting using a 200µL Gilson Pipette. 350µL DNA Dilution Buffer was added and the sample was placed at 70ºC for 3min using a heating block (QBT1/Grant). The sample was centrifuged at 130000rpm for 10min. The lysate was transferred to a new 1.5mL Eppendorf tube and 200µL of 95% ethanol + 5% dH_2O was added and mixed. The solution was transferred to the spin columns and spun at 130000rpm for 1min. The collected nucleic acids were washed in 600µL RNA Wash Solution and spun at 130000rpm for 1min. The DNA was degraded using 5µL DNase I in 40µL Yellow Core Buffer and 5µL 0.09M Manganese chloride ($MnCl_{2)}$ for 15min at room temperature. The degradation was terminated using 200µl DNase Stop Solution and the sample was spun at 130000rpm for 1min. The sample was washed with 600µL RNA Wash Solution and spun at 130000rpm for 1min. The sample was additionally washed with 250µL RNA Wash Solution and spun at 130000rpm for 2min. The RNA was eluted in 100µL dH_2O and spun down into a fresh 1.5µL Elution tube at 130000rpm for 1min. RNA quantification was carried out using a 1:25 dilution (40µL RNA + 960µL dH_2O). For RNA quantification the absorbance was measured at 260nm and quantified as 1 unit absorbance equalling 40µg RNA per mL. 200ng RNA was carried into each cDNA synthesis reaction using M-MLV reverse transcriptase (M1701/Promega). Briefly, each cDNA synthesis reaction contained: 1µL Random Primers, 2µL dNTPs, 0.5µL RNase Inhibitor, 4µL MLV Buffer, 1µL MLV-RT, 200ng RNA, adjusted amounts of dH_2O. Total volume was 20µL. Random Primers, RNA and dH_2O were initially mixed and placed at 70ºC for 5min before the remaining components were added. The cDNA synthesis was carried out at 37ºC for 1hr followed by 10min at 75ºC using a PRC machine (TC-312/Techne). PCR reactions were performed using GoTaq DNA polymerase (M3175/Promega) with 35 cycles of (94ºC:1min, 55ºC:1min, 72ºC:1min). β-actin was used as a loading and DNA-content control. PCR reactions were run on a 1% agarose gel (15517-

014/Invitrogen):1xTris Borate EDTA (0.089M Tris borate, 2mM EDTA as specified by SIGMA) (T-3913/SIGMA).

2.10 Statistical analysis

Comparisons of two sets of data were analysed using a one-tailed Student's unpaired t-test between control samples and experimental samples. Data is expressed as the mean ± Standard Error of the Mean. Significance was determined as $p<0.05$.

Chapter 3

Rac1-GTPase, a regulator of renal morphogenesis

3.1 Introduction

3.1.1 Overview

The development of the kidney requires intricate mechanisms to regulate the morphogenesis of several different structures. The molecular components and the mechanisms that regulate these processes remain largely unknown. This thesis contains two results chapters that describe experiments aimed at determining what these processes are. In this chapter, Chapter 3, I focus on the protein Rac1-GTPase (Rac1) and I attempt to elucidate whether Rac1 has a function during nephron formation, ureteric bud differentiation, branching morphogenesis and kidney growth. In Chapter 4 I present work that describes the role of the RhoA-GTPase effector Rho-kinase and in a similar manner to in Chapter 3, also try to show what the role is for Rho-kinase in kidney development. Initially, Rac1 and Rho-kinase received equal attention, but as the Rho-kinase branch was considered more interesting and ultimately more publishable, I decided to concentrate on that. As a result of this, some aspects of Chapter 3 were not pursued to the level of depth that is sometimes presented in Chapter 4.

Rac1 has been shown to be a very versatile protein that is important for numerous cellular functions such as motility (Ridley et al 1992; Nobes and Hall 1995; Machesky and Hall 1997; Nobes and Hall 1999), tubulogenesis (Montesano, Schaller, and Orci 1991; Montesano et al 1991; Pollack, Runyan, and Mostov 1998; Rogers et al 2003) and proliferation (Minden et al 1995; Westwick et al 1997; Gu et al 2003). Rac1 has also been linked to the planar cell polarity pathway in vivo, regulating convergent extension in Xenopus (Habas, Dawid, and He 2003). Because Rac1 regulates such a wide array of processes, I will highlight important functions in the introduction to ensure that the results sections are as focussed as possible. Each introductory section below is labelled with the Rac1-function described therein. Some of the functions described in the introduction are not directly demonstrated in the results but they are important to consider.

3.1.2 Rac1 and mechanisms of motility

Cellular motility is a possible mechanism that accounts for branching morphogenesis of the ureteric bud and may also be important for nephron formation during the condensation of pretubular aggregates. In the mammary glands, collective cell migration is necessary for the formation of new ducts and branching, a process that is Rac1 dependent (Ewald et al 2008). Evidence suggesting a role for motility and a tight regulation of actin filaments during ureteric bud branching has also emerged (Michael and Davies 2004; Watanabe and Costantini 2004; Michael, Sweeney, and Davies 2005; Meyer et al 2006; Kim and Dressler 2007). Rac1 regulates cellular motility by acting as an organiser for the cytoskeleton (Ridley and Hall 1992; Ridley et al 1992; Nobes and Hall 1995; Machesky and Hall 1997; Nobes and Hall 1999). Cellular motility is produced by the nucleation of actin filaments which in turn is regulated by a multiprotein-complex Arp2/3 (Welch, Iwamatsu, and Mitchison 1997; Robinson et al 2001). The Arp2/3 complex is continuously bound to the WASP family Verproline homologous proteins (WAVE) complex that becomes active upon recruitment to the plasma membrane by Rac/Nck/Abi1 (Innocenti et al 2004), reviewed by Bompard and Caron (Bompard and Caron 2005). The actin nucleation at the leading edge of cells produces the force which the cells use to move, reviewed by Stossel (Stossel 1993). A mechanism of 'elastic Brownian ratchet' has been proposed by Mogilner and Oster, where it is suggested that the elasticity of actin filaments and the plasma membrane, in conjunction with the thermal motion by both these structures, allow the actin filaments to 'sway' away and towards the plasma membrane (Mogilner and Oster 1996; Mogilner and Oster 2003). This allows actin polymerisation to occur when the filament is in an 'away' position and consequently, upon swaying back into the 'towards' position, the actin filament will be longer and hence force the plasma membrane forwards. The net force generated by numerous actin filaments would be sufficient for cellular motion (Mogilner and Oster 1996; Mogilner and Oster 2003).

3.1.3 Rac1 in cystogenesis and tubule formation

Rac1 is important for cell cystogenesis and the formation tubules from such cysts (Montesano, Schaller, and Orci 1991). In the immediate context, cystogenesis and tubule formation refer to the formation of epithelial cysts and tubules from an existing epithelium rather than the cystogenesis that is seen during the mesenchymal-to-epithelial transitions which give rise to the nephrons. The formation of new tubules from an existing epithelium, is a recurring phenomenon during embryonic development that is exemplified in the kidneys and the lungs (Miettinen et al 1997; Michael and Davies 2004). Tubule formation can be studied using several different culture systems such as epithelial cell cultures in collagen gels, isolated ureteric buds in matrigel and whole in vitro embryonic kidney and lung cultures. Rac1 has been shown to be directly involved in the in vitro formation of tubular structures from MDCK cell cysts (Rogers et al 2003). When cultured in a three-dimensional collagen type-I assay system, MDCK cells form cysts that, when stimulated with hepatocyte growth factor (HGF), form branched tubules (Montesano, Schaller, and Orci 1991; Montesano et al 1991). This formation of tubules from MDCK cysts follows a specific pattern of four stages (extension, chain, cord and tubule) which are characterised by modifications of cell-cell adhesion and apical/basolateral polarity (Pollack, Runyan, and Mostov 1998). Rac1-GTPase has been demonstrated to be important for MDCK cystogenesis and tubulogenesis (O'Brien et al 2001). Cysts derived from MDCK cells with a dominant-negative *Rac1* mutation, *rac1N17*, (see Table 3.1 for Rho-GTPases mutants), lacked the ability to polarise normally. The apical surface, facing the lumen, normally marked by glycoprotein 135 (gp135) and microvilli, was lost and gp135 was redistributed to the fringes of the cyst. The basolateral markers E-cadherin and p58 were found on all cell surfaces unlike wild type cysts where they are excluded from the apical surface (O'Brien et al 2001). *Rac1N17* cysts lacked normal laminin assembly basally, a phenotype that could be rescued by the addition of laminin (O'Brien et al 2001). This abnormal laminin staining observed basally was speculated to be a result of the decreased amount of α3β1 integrin which was detected. The authors hypothesised that there may be a signalling pathway where Rac1 stimulates α3β1 integrin, resulting in laminin

assembly basally, which in turn provides a cue for the basal pole to be specified (O'Brien et al 2001). Importantly for this discussion, the MDCK cyst that formed with inversed polarity failed to form tubules (Rogers et al 2003). MDCK cell lines have been produced that carry inducible dominant negative and constitutively active *rac1*, *rhoA* and *cdc42* genes (Table 3.1) as were used by Rogers and colleagues (Rogers et al 2003).

Gene name	**Dominant negative mutant form**	**Constitutively active mutant form**
rac1	rac1 T=>N17	rac1 G=>V12
cdc42	cdc42 T=>N17	cdc42 G=>V12
rhoA	rhoA T=>N19	rhoA G=>V14

Table 3.1 Rho-GTPase mutant forms

Rogers and colleagues focussed on the roles of Rac1, RhoA and Cdc42 GTPases during cystogenesis and tubulogenesis (Rogers et al 2003). Only *rhoAV14* completely disrupted cystogenesis and tubulogenesis whereas the other mutants exhibited lesser effects (Rogers et al 2003). *rhoAN19*, *rac1N17*, *cdc42V12* and *cdc42N17* MDCK cell cysts all exhibited inversions of cyst polarity, with the effects in *cdc42N17* and *cdc42V12* MDCK cell cysts being the most prominent (Rogers et al 2003). Of those mutant cell lines which formed cysts, only *rac1N17* MDCK cell cysts inhibited tubulogenesis (Rogers et al 2003). MDCK cells carrying the *rac1V12* gene were however capable of tubulogenesis in the absence of HGF (Rogers et al 2003). These studies indicate a critical role for *Rac1*, *RhoA* and *Cdc42* expression for in vitro cystogenesis and tubulogenesis. It is arguable to what extent that data from such in vitro cell culture studies can be extrapolated to a whole organ. Although, as mentioned in the previous paragraph, Rac1 has been demonstrated to be necessary for the formation of new tubules during mammary gland duct formation via the process of migration (Ewald et al 2008). These results underline the importance of carrying out additional studies to determine whether Rac1 might regulate similar processes in the kidney.

3.1.4 Rac1, a regulator of proliferation

Rac1 has previously been shown to have a role during kidney development (Osafune et al 2006). It was reported that Rac1, possibly acting via a Jun N-terminal Kinase (JNK) planar cell polarity pathway, regulates the size of renal progenitor cell cultures (Osafune et al 2006). A possible mechanism for this control would be through the regulation of proliferation. Rac1 has been shown to be an important regulator of proliferation and cell cycling (Minden et al 1995). The life cycle of a cell is characterised by several phases consisting of: Growth-phase 1 (G1), DNA-synthesis (S), Growth-phase 2 (G2), Mitosis (M) and a quiescent phase (G0) which is not directly involved in cellular proliferation (Johnson and Walker 1999). Cell cycle progression and entry from the G0-phase is tightly regulated due to the potential hazards of uncontrolled cellular proliferation. Rac1 has been demonstrated to regulate cell cycling in a number of different cell types in vitro. Gu and colleagues studied the roles of Rac1 and Rac2 for haematopoietic stem/progenitor (HSC/P) cells using a conditional $Rac1^{flox/flox}$ (Gu et al 2003). Their findings strongly suggest that Rac1 is necessary for HSC/P cells' growth and progression of the cell cycle. $Rac1^{-/-}$ cells failed to enter S, G2 or M phase and this was likely due to an absence of cyclin D1 protein, as compared to normal HSC/P cells. Cyclin D1, D2 and D3 are important for G0 to S-phase progression, as is extensively reviewed by Johnson and Walker (Johnson and Walker 1999). Cyclin D1 is known to activate Cyclin-dependent kinases 4 and 6 (Cdk4 and Cdk6) and to phosphorylate retinoblastoma tumour suppressor protein (Rb) (Johnson and Walker 1999). This phosphorylation disrupts the binding of Rb to the transcription factor family E2F which are essential for progression from G0-phase to S-phase (Johnson and Walker 1999). Rac1 can bind PAK and induced the transcription of cyclin D1 (Westwick et al 1997). Rac1 can activate JNK and p38MAPK, which in turn induces the transcriptional activity of c-Jun which is important for cell-cycle progression (Westwick et al 1997).

Plenty of research supports the theory that Rac1-stimulated proliferation can be a result of adhesion-regulated proliferation (Mettouchi et al 2001). Human umbilical vein endothelial cells (HUVECs) display adhesion dependent proliferation rates

where HUVECs cultured on fibronectin proliferate and enter S-phase, whereas HUVECs cultured on laminin-1 enter growth arrest (Mettouchi et al 2001). Cell culture medium supplemented with the growth factors, basic fibroblast growth factor (bFGF) and insulin cause the cells to apoptose (Mettouchi et al 2001). This difference in cellular behaviour was shown to be a result of different integrins. HUVECs express *α5β1 integrin* and binding of α5β1 to fibronectin triggered the activation of Rac through a signalling pathway that included a number of proteins such as Shc, FAK, PI-3-K and SOS (Mettouchi et al 2001). This activation was shown to result in increased translation of cyclin D1 protein (Mettouchi et al 2001). Importantly, the amounts of cyclin D1 were found to be the same in HUVECs cultured on fibronectin and on laminin-1 (Mettouchi et al 2001). This was explained by the PAK/p42/p44MAPK pathway still being active and as a result regulating cyclin D1 (Mettouchi et al 2001). On the other hand, Moore and colleagues tested the effects of injecting a dominant negative *Rac1 (Rac1N17)* adenovirus into Rat2 fibroblast cells (Moore et al 1997). They found that, in an adenovirus-*Rac1N17* concentration-dependent manner, cells accumulated in G2/M transition. The highest percentage of cells in G2M transition was found in those cultures which had received the highest concentration of the adenovirus (Moore et al 1997). Further tests by Moore and colleagues using injections of dominant negative *RhoA* and *Cdc42* adenoviruses did not have the same effects (Moore et al 1997).

It is clear that Rac plays an important role in the cell cycle and proliferation at several different levels. An interesting prospect would be that Rac1 regulates kidney proliferation via the MAP-Kinase (MAPK) signalling cascades. MAP-Kinases p42MAPK and p44MAPK, also known as ERK2 and ERK1, are important for re-entry from the G0 to the S-phase (Pages et al 1993). In the absence of activated p42/44MAPK the cells were arrested in the G0-phase (Pages et al 1993). Lavoie and colleagues later discovered that the regulation of cell cycle progression was regulated by p42/44MAPK and another MAPK; p38/HOGMAPK (Lavoie et al 1996). Activation of the p42/44MAPK pathway resulted in the expression of cyclin D1 and G0 to S-phase progression. Activation of p38/HOGMAPK, on the other hand, inhibited cyclin D1 transcription resulting in cell cycle arrest. Cyclin D1 expression

can also be negatively regulated by transforming growth factor β1 (TGF-β) signalling via the MAPK kinase kinase (MKK) transforming growth factor-β activating kinase-1 (TAK1) which activates the MKK3/6-P38MAPK pathway (Terada et al 1999). This leads to cell cycle arrest (Terada et al 1999). These authors also showed cyclin D1 to be positively regulated by the p42/44MAPK pathway (Terada et al 1999).

In the kidney both p42MAPK and p44MAPK are expressed and activated in both the ureteric bud and in the metanephric mesenchyme of E11 kidneys (Fisher et al 2001). Inhibition of MAP kinase kinase 1 (MEK1) and to a lesser extent MAP kinase kinase 5 (MEK5) resulted in several changes in kidney morphology and cell behaviour (Fisher et al 2001). E11.5 kidneys cultured in vitro in the presence of the MEK inhibitor exhibited long un-branched ureteric buds where tubule elongation was preserved but branching was clearly disturbed (Fisher et al 2001). The formation of new branches was abolished by MEK inhibitor concentration dependent manner (Fisher et al 2001). Normal apical localisation of actin at the apical surface of cells in the ureteric bud tips was found to be reduced or absent. Significantly, the proliferation rate was markedly reduced in the ureteric bud (Fisher et al 2001). Furthermore, Fisher and colleagues explored upstream regulators of p42MAPK and p44MAPK activation and found that in the kidney, GDNF is important for activation of these MAP kinases (Fisher et al 2001).

Rac1 is a regulator of MAPKs. In vitro experiments have shown that dominant negative *Rac1* could effectively inhibit JNK activation and constitutively active *Rac1* had a strong opposite effect (Coso et al 1995; Minden et al 1995). This connection between Rac1 and JNK could provide the direct pathway regulating renal progenitor cell cultures (Osafune et al 2006). The dominant negative and constitutively active *Rac1* mutants did not have any effect on the activation of the p42/p44MAPK pathway (Coso et al 1995; Minden et al 1995). Rac1 also can activate p38MAPK (Minden et al 1995). Interestingly, contrary to previous reports, it has also been shown that Rac1 can activate the p42MAPK pathway (Eblen et al 2002). The activation of p42MAPK was promoted by cell adhesion to fibronectin, again

suggesting a role for Rac-PAK in adhesion regulated cell cycling (Eblen et al 2002). The possibility that Rac1 regulates proliferation rates in the kidney needs to be investigated in order to better understand possible mechanisms of size regulation.

3.1.5 Rac1 and the kidney

Rac1 is present in the majority of embryonic kidney compartments in the mouse metanephric kidney (www.GUDMAP.org). Rac1 has been suggested to have a role during kidney development where Rac1 affects a planar cell polarity pathway, possibly acting through Jun N-terminal Kinase (JNK) (Osafune et al 2006). Osafune and colleagues showed that it is possible to culture, in vitro, what they identified as embryonic nephron progenitor cells (Osafune et al 2006). In their results they described that, Rac1 is capable of regulating the size of such cultures (Osafune et al 2006). In whole kidney cultures it was demonstrated that, JNK can act as a regulator for the size of these kidneys (Osafune et al 2006). The disruption of JNK signalling in the nephron progenitor cultures resulted in these cells failing to express markers for differentiation (Osafune et al 2006). This previous data demonstrated that Rac1 might have a role in kidney development but it did not present a mechanism accounting for how Rac1 controls nephron progenitor culture sizes or whether Rac1 might be an upstream regulator of the JNK regulation.

In this chapter I will discuss several experiments carried out in order characterise the role of Rac1 during branching morphogenesis and nephrogenesis. In order to regulate Rac1 activity I used a commercially available Rac1 inhibitor (NSC23766), which blocks Rac1 activation (Gao et al 2004). Point mutation experiments in several regions of Rac1 have identified residues important for Rac1-activation by GEF and GEF recognition specificity (Gao et al 2004). Through the introduction of point mutations into specific regions, referred to as switch I, β2/β3 and switch II of Rac1, it was determined that residues 53-72 of Rac1 were important for Rac1/GEF recognition (Gao et al 2001). In particular, residue Trp56 in the region known as β3 was crucial (Gao et al 2001; Gao et al 2004). Trp56 was necessary for Rac1 to

recognise GEFs such as Trio, T-cell lymphoma invasion and metastasis 1 (Tiam1) and GEF-H1 (Gao et al 2001; Gao et al 2004). When Cdc42F56W mutants were generated, these showed similar binding to GEFs as Rac1 (Gao et al 2001; Gao et al 2004). Through a structural based virtual screening, chemical compounds were identified that were capable of interacting with this region and compound NSC23766 was isolated (Gao et al 2004). At 50µM, NSC23766 was found to inhibit Tiam binding to Rac1 by 50% and TrioN binding to Rac1 at a similar amount (Gao et al 2004). Significantly, at this concentration the authors were unable to detect any effects of the drug on RhoA or Cdc42 binding to their GEFs (Gao et al 2004). The authors specified several reason why they argue in favour of NSC23766 acting in a Rac1 specific manner and not on other targets (Gao et al 2004). Firstly, NSC23766 specifically inhibited Rac1 activation by Rac1-specific GEFs and Gao and colleagues did not detect any interference of the drug with GEFs that are not Rac1 specific or that interact with the other Rho-GTPases RhoA and Cdc42 (Gao et al 2004). Secondly, the authors did not detect any effects of NSC23766 on constitutively active Rac1 mutants, suggesting that the effects are specific on the activation of Rac1 (Gao et al 2004). NSC23766 was thus chosen as a good method to inhibit Rac1 in kidneys.

3.2 Results

3.2.1 Inhibition of Rac1-GTPase reduces ureteric bud branching.

In order to determine whether Rac1-GTPase (Rac1) plays a role during kidney development, Rac1 inhibitor NSC23766 was used.

To establish whether Rac1 plays a role during kidney development, whole kidney cultures were set up and treated with several different concentrations of NSC2376. The highest concentration used was equivalent to that which has been published for experiments on mammary gland development (Ewald et al 2008). The lowest concentration used was that specified to inhibit 50% of Tiam-Rac1 binding in NIH

3T3 cells (Gao et al 2004). The amount/degree of branching was used as indicator whether Rac1 inhibition affected ureteric bud branching. The degree of branching was assessed by counting the number of ureteric bud tips i.e. the terminal regions of the ureteric bud tree that are most distal to the ureter.

Whole kidney cultures were set up as specified in Chapter 2 and maintained for 72hrs in control conditions or in 50µM, 75µM or 100µM NSC23766. For the remainder of the thesis I will interchangeably refer to experimental controls as simply "controls" or to samples in "control conditions". Alternatively, the abbreviation for complete kidney culture medium (CKCM) is also used. Controls contained a volume of carrier equal to that used in the experimental conditions. To count the ureteric bud tips, the cultures were incubated with anti-Calbindin D-28K which was detected with a fluorescent secondary anti-mouse antibody that allowed for immunofluorescent visualisation of the entire ureteric bud. Calbindin D-28K protein is only found in the ureteric bud in the kidney at this stage (Davies 1994). Quantification of ureteric bud tips showed that compared to controls (Fig.3.1A,E) cultures in 50µM of NSC23766 displayed only 60% of ureteric bud tips (Fig.3.1B,E) n=10,10 kidneys, p=0.00034. At 75µM NSC23766 (Fig.3.1C,E) the decrease is marginally greater as they have only 58% of the number of tips in control conditions, n=9 kidneys, p=0.00057. At the higher concentration of 100 µM (Fig.3.1D,E), the cultures had ~48% of the number of ureteric bud tips seen in controls, n=9 kidneys, p=0.000006. These reductions in branching were statistically confirmed using separate unpaired one-tailed Student's t-test comparing the number of ureteric bud tips in the kidneys in each experimental culture condition against the control kidneys. The decrease in branching was not accompanied by abnormal branching morphology or abnormal epithelial morphology compared to controls (Fig.3.1A-D). These results are in agreement with previously published data, which using a dominant negative Rac1, demonstrated that Rac1 positively regulates renal progenitor cell culture size (Osafune et al 2006). They showed that cultures with the dominant negative Rac1 were only 46% of the size of control cultures. This data thus shows that inhibition of Rac1 not only affects the renal progenitor cell population but also negatively affects an important process of kidney development, namely ureteric bud branching. The

concentration chosen for further experiments was 75μM NSC23766 which is lower than the 100μM NSC23766 utilised in order to study mammary gland development (Ewald et al 2008).

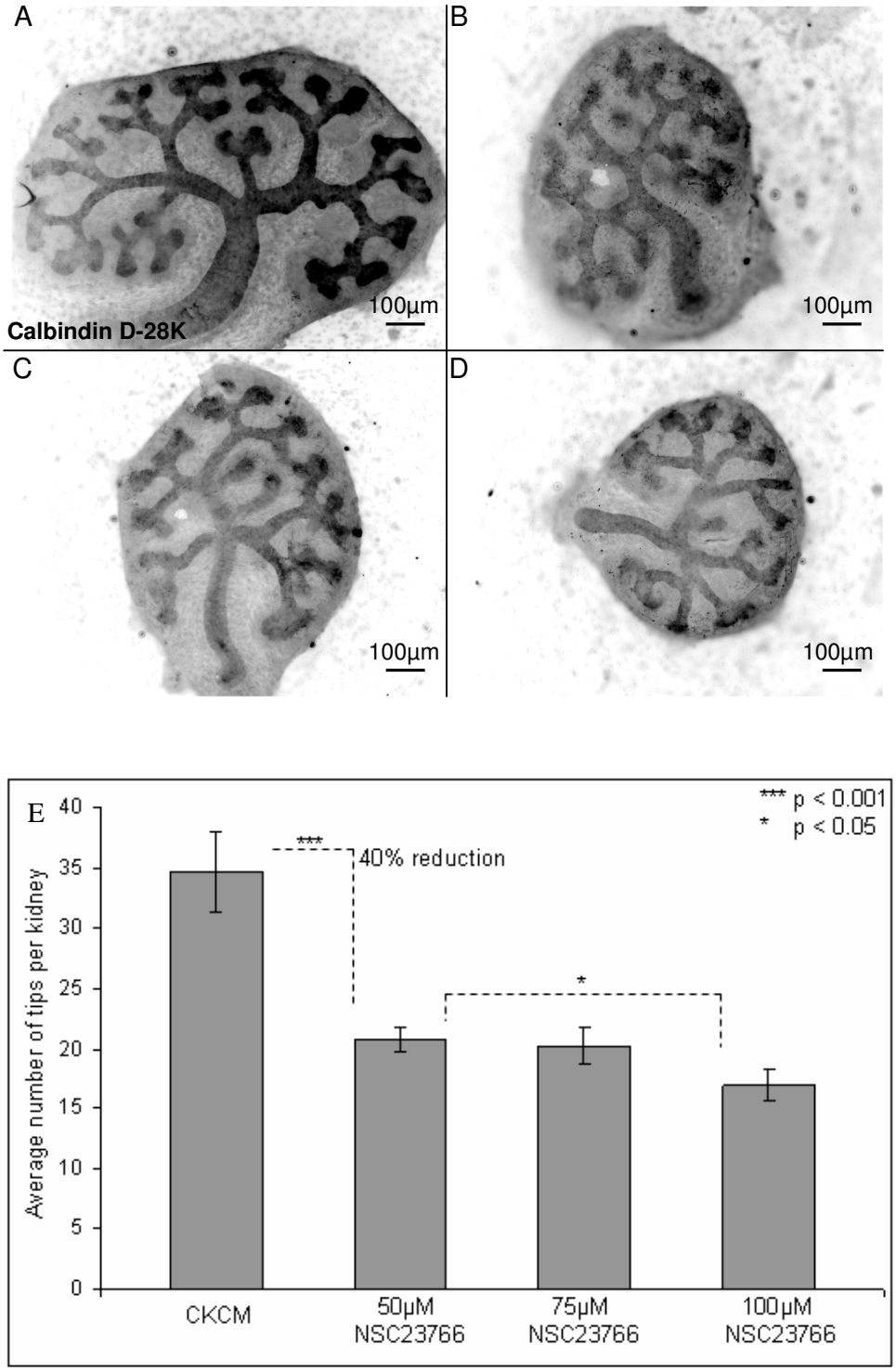


Fig.3.1 The effects of Rac1 inhibition on kidney branching

Fig.3.1 shows kidneys that were cultured in CKCM or CKCM containing 50µM, 75µM or 100µM NSC23766 for 72hrs and then fixed in methanol and stained for Calbindin D-28K-mouse which was detected with an anti-mouse-FITC secondary antibody. (A) Kidney cultured in CKCM (n=11). (B)

Kidney cultured in 50µM NSC23766 in CKCM (n=11). (C) Kidney cultured in 75µM NSC23766 in CKCM (n=10). (D) Kidney cultured in 100µM NSC23766 in CKCM (n=10). (E) Bar chart showing the mean values ± SEM of the number of ureteric bud tips for kidneys cultured in each culture condition. Data was analysed separately using unpaired one-tailed Student's t-tests. T-test p-values for: CKCM vs. 50µM NSC23766 p= 0.00034, 50µM NSC23766 vs. 100µM NSC23766 p = 0.014.

3.2.2 Inhibition of Rac1-GTPase decreases overall kidney growth and branching in a reversible manner.

The effects that the Rac1 inhibitor NSC23766 had on ureteric bud branching required additional experiments to characterise these results. Firstly, it was necessary to determine whether the effects of the inhibitor were permanent or if the withdrawal of the inhibitor would allow for the kidney to revert to normal branching. Secondly, I investigated whether the reduced branching could be a result of an overall decrease in kidney growth since the branching appeared morphologically normal. The degree of kidney growth can be estimated by measuring the diameter of the kidneys in culture. Kidneys are not circular, so the diameter will vary depending on where the measurements are taken. To take this into account the diameter was derived as an approximation by measuring the largest and smallest diameter from the centre of the first ureteric bud bifurcation to the kidney periphery. The diameter used was the mean of these two values. The degree of branching was again quantified using the average number of ureteric bud tips per kidney. These two measurements were both used to determine whether the effects of the Rac1 inhibitor were reversible.

Four sets of whole kidney cultures were set up and cultured as follows:

Set	**Conditions**	**Time (hours)**
A	Control conditions	72hrs
B	75µM NSC23766	72hrs
C	75µM NSC23766	72hrs
--->	+ control conditions	72hrs
D	75µM NSC23766	72hrs
--->	75µM NSC23766	72hrs

Table 3.1 Culture conditions to test reversibility of effects by NSC23766.

In order to test for reversibility, cultures were initially maintained in their respective conditions for 72hrs. At that time point set A (Fig.3.2A) and set B (Fig.3.2B), were fixed. The other two set of cultures (set C and set D), were maintained for an additional 72hrs in control conditions, set C (Fig.3.2C), or in fresh 75µM NSC23766

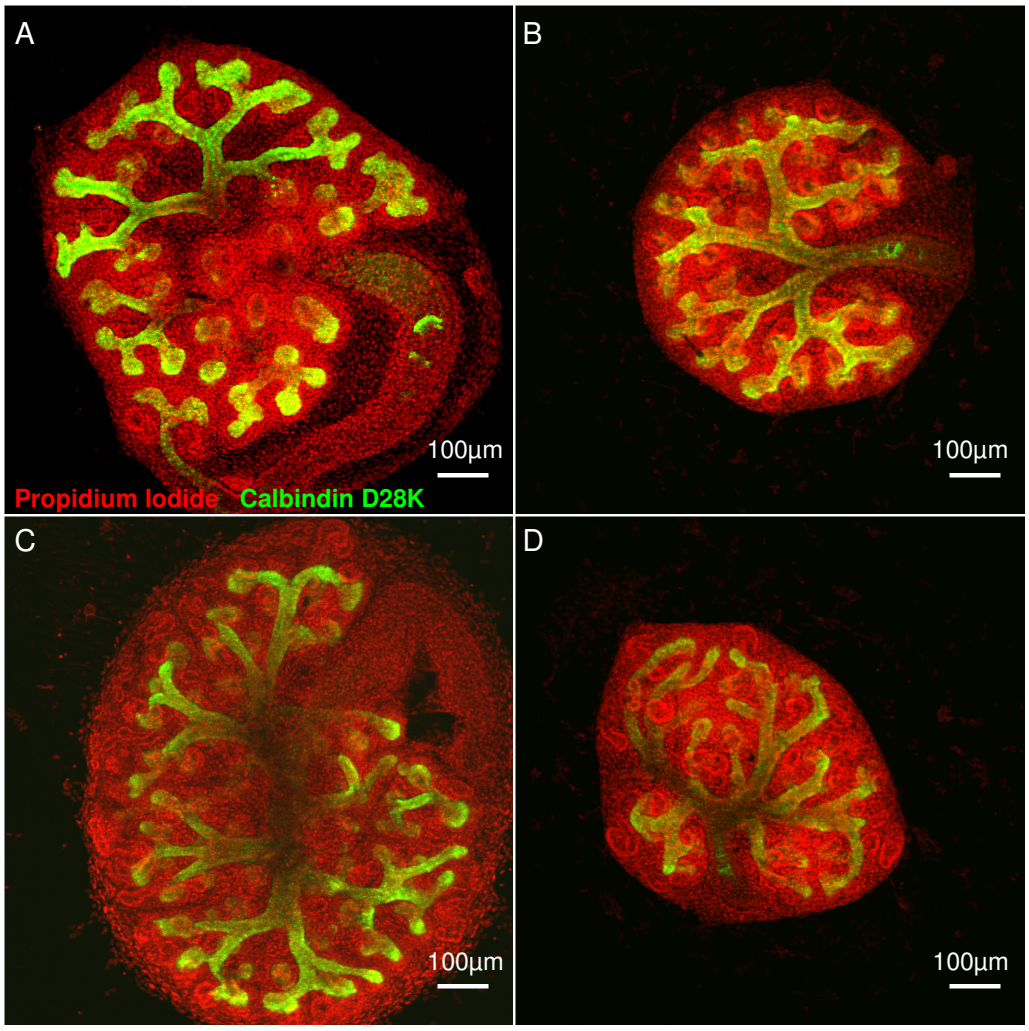
medium, set D (Fig.3.2D). To determine whether the effects were reversible, set A was compared with set C. The data was analysed using separate unpaired one-tailed Student's t-tests. There was no significant difference in the average number of ureteric bud tips between these cultures, n=7,6 kidneys, p=0.32 (Fig.3.2E). In addition, there was no difference in the average diameter of the kidneys in these sets, p=0.12 (Fig.3.2F). The control set B, that demonstrates the effectiveness of the Rac1 inhibitor during the initial 72hrs displayed a reduction in branching compared to set A as in previous experiments, (Fig.3.2E), n=7,7 kidneys, p=0.016, thus demonstrating the Rac1 inhibitor was effective for the first 72hrs in these experiments. The average diameter of SetB was 70% of that in SetA p=0.00019 (Fig.3.2F).

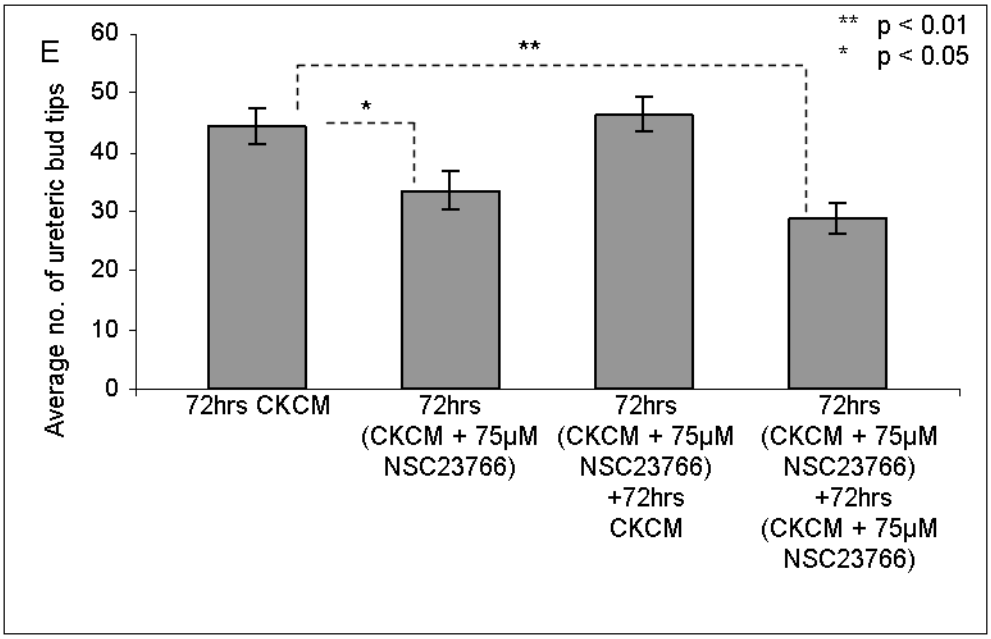
Those kidneys in 'reversed' conditions, set C, had significantly more tips (Fig.3.2E,) than those that were cultured in inhibitor for a total of 144hrs, set D (Fig.3.2D) n=6,6 kidneys, p=0.00067. They also displayed increased average diameter (Fig.3.2F), p=0.013. The same was seen when comparing the 'reversed' conditions, set C, with those kidneys cultured for 72hrs in inhibitor, set B: average ureteric bud tips (Fig.3.2E) n=6,7 kidneys, p=0.006; average diameter (Fig.3.2F) (p=0.00026). These results demonstrate that the 'recovery' of set C displays real differences to kidneys that were not permitted to recover, set D, and have subsequent to the transfer to control conditions now branched to a degree much higher than at 72hrs.

It was also confirmed that kidneys cultured in control conditions exhibit significant growth and branching past the initial 72hrs and until 144hrs, n=4,4 kidneys, p=0.0016 (Fig.3.2G). This was confirmed to ensure that the significant differences described above were indeed direct effects of the drug NSC23766 and not results due to limitations of the culture system beyond 72hrs of culture.

This data shows that the addition of NSC23766 to kidney cultures results in replicable, quantifiable effects where Rac1 inhibition leads to reduced branching and reduced overall growth. The inhibiting effects are reversible through the removal of the drug. The complexity of kidney development and the crosstalk of inductive and inhibitory signals (Saxen 1987) allows for several hypotheses to be investigated. The

multitude of Rac1 functions also adds additional possibilities for how Rac1 inhibition might produce the above described effects.


A
B
C
D
100µm
100µm
100µm
100µm
Propidium Iodide
Calbindin D28K


E
Average no. of ureteric bud tips
60
50
40
30
20
10
0
** p < 0.01
* p < 0.05
**
*
72hrs CKCM
72hrs (CKCM + 75µM NSC23766)
72hrs (CKCM + 75µM NSC23766) +72hrs CKCM
72hrs (CKCM + 75µM NSC23766) +72hrs (CKCM + 75µM NSC23766)

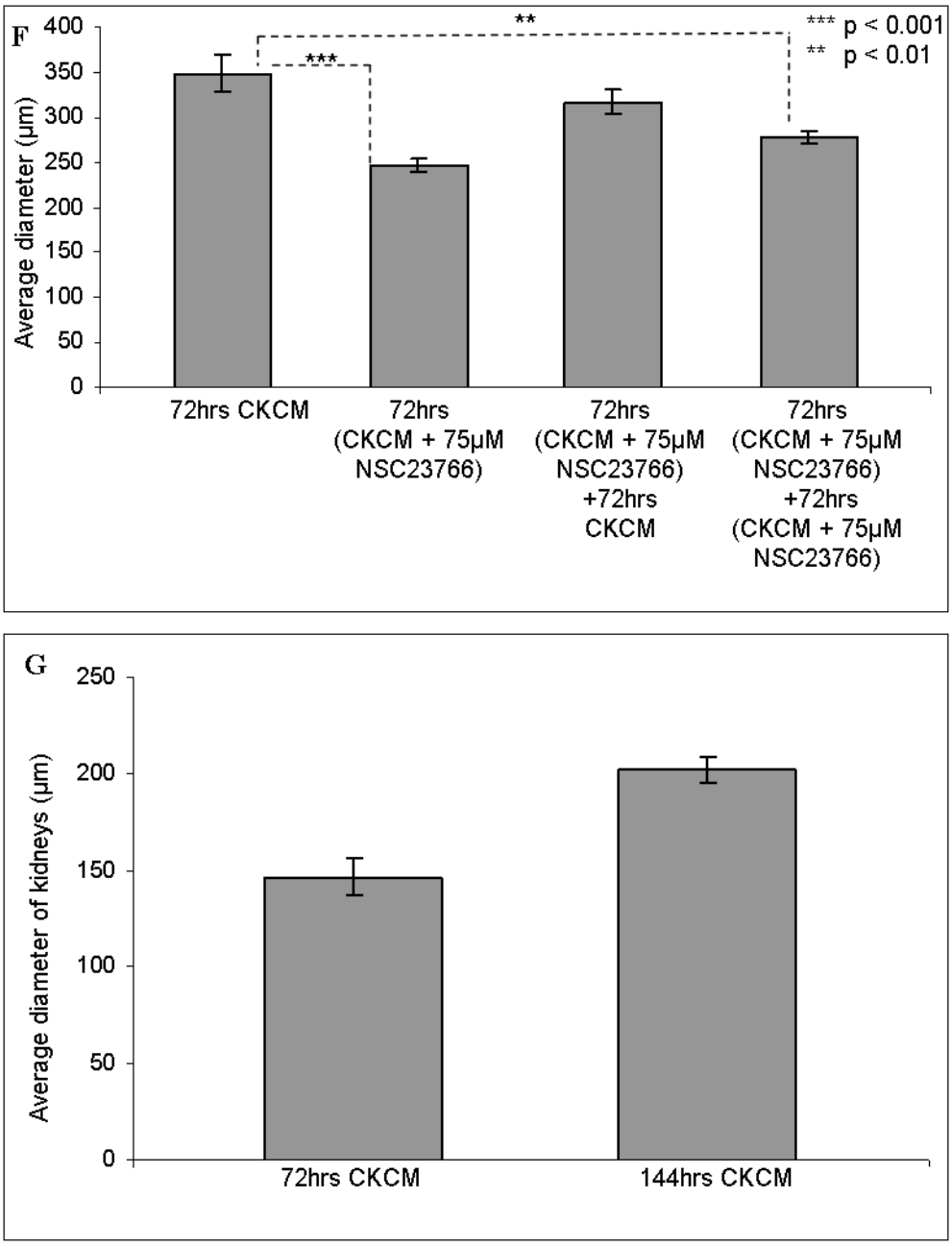


Fig.3.2 The effects of Rac1 inhibition on kidney branching, growth and the reversibility of these effects

Fig.3.2 (A-F) shows the effects of and the reversibility of the effects by NSC23766 on growth and branching morphogenesis. (A) Kidney cultured for 72hrs in CKCM (n=7). (B) Kidney cultured for 72hrs in CKCM with 75µM NSC23766 (n=7). (C) Kidney cultured for 72hrs in CKCM with 75µM NSC23766 and then transferred to CKCM for an additional 72hrs (n=6). (D) Kidney cultured for 72hrs in CKCM with 75µM NSC23766 medium and then transferred to CKCM also with 75µM

NSC23766 for an additional 72hrs (n=6). Kidneys were stained for Calbindin D-28K which was detected with anti-mouse-FITC. Kidneys were also stained with Propidium Iodide (TRITC). (E) The graph shows the mean values ± SEM of the number of ureteric bud tips for each treatment category. P-values were derived separately using an unpaired one-tailed Student's t-test. T-test p-values for: Set A vs. Set C p=0.32, Set A vs. Set B p=0.016, Set C vs. Set D 0.00067, Set B vs. Set C p=0.006. (F) The graph shows the mean values ± SEM of the diameter of kidneys for each treatment category. T-test p-values for: Set A vs. Set C p=0.12, Set A vs. Set B p=0.00019, Set C vs. Set D 0.013, Set B vs. Set C p=0.00026. (G) The bar chart shows the mean values ± SEM of the diameter of kidneys cultured in CKCM for 72hrs n=4 and 144hrs n=4, p=0.00016.

3.2.3 Inhibition of Rac1-GTPase does not, markedly affect the actin cytoskeleton neither in the ureteric bud nor in surrounding mesenchyme.

The distribution of actin filaments within the ureteric bud has been shown to be regulated by several proteins and pathways. Inhibition of the RhoA pathway by (use of) Rho-Kinase inhibitor Y-27632 results in the loss of apical localisation of actin in the ureteric bud tips (Michael, Sweeney, and Davies 2005). The same effect was seen when MEK1 and the p42/44MAPK pathway was inhibited (Fisher et al 2001). Rac1 is closely connected to both the RhoA-pathway (Habas, Dawid, and He 2003; Rosenfeldt et al 2006) and it is also possibly connected to the p42/44MAPK pathway (Eblen et al 2002). It is therefore necessary to investigate whether Rac1 might also regulate this apical actin localisation.

Kidney cultures were set up in control conditions or in 75µM Rac1 inhibitor NSC23766 in order to determine what effect inhibition of Rac1 had on the actin cytoskeleton both in the ureteric bud and in the surrounding metanephric mesenchyme. Cultures were fixed after 72hrs and stained using FITC-conjugated phalloidin, as previously used by Michael, Sweeney and Davies (Michael, Sweeney, and Davies 2005). This allowed for high-resolution confocal scanning of the samples visualising the actin cytoskeleton. Unlike RhoA, inhibition of Rac1 in kidney cultures with 75µM NSC23766 had no discernable effects on the apical actin cytoskeleton in the ureteric bud tips. Control kidneys (Fig.3.3A) and those kidneys cultured in 75µM NSC23766 (Fig.3.3B) displayed cells with highly structured actin cytoskeletons. The ureteric bud tips show slightly elevated localisation of actin to the apical and basal surfaces compared to stalk regions. The metanephric mesenchyme surrounding the tips, the cap mesenchyme, displays highly organised actin filaments. These were also unaffected by the inhibition of Rac1.

The roles of Rac1 and RhoA are evidently different in terms of their regulation of the actin cytoskeleton in the kidney. However, as Rac1 affects the formation of cellular lamellipodium (Ridley et al 1992) which is more of a micro-structure, it is not expected to affect the localisation of large bundles of actin filaments in the same way as the RhoA pathway. Nevertheless, as Rac1 has been shown to both inhibit and activate RhoA in different cell types (Nobes and Hall 1995; Rosenfeldt et al 2006) it

was a possibility that Rac1 inhibition would have an effect on structures proven to be regulated by the RhoA pathway.

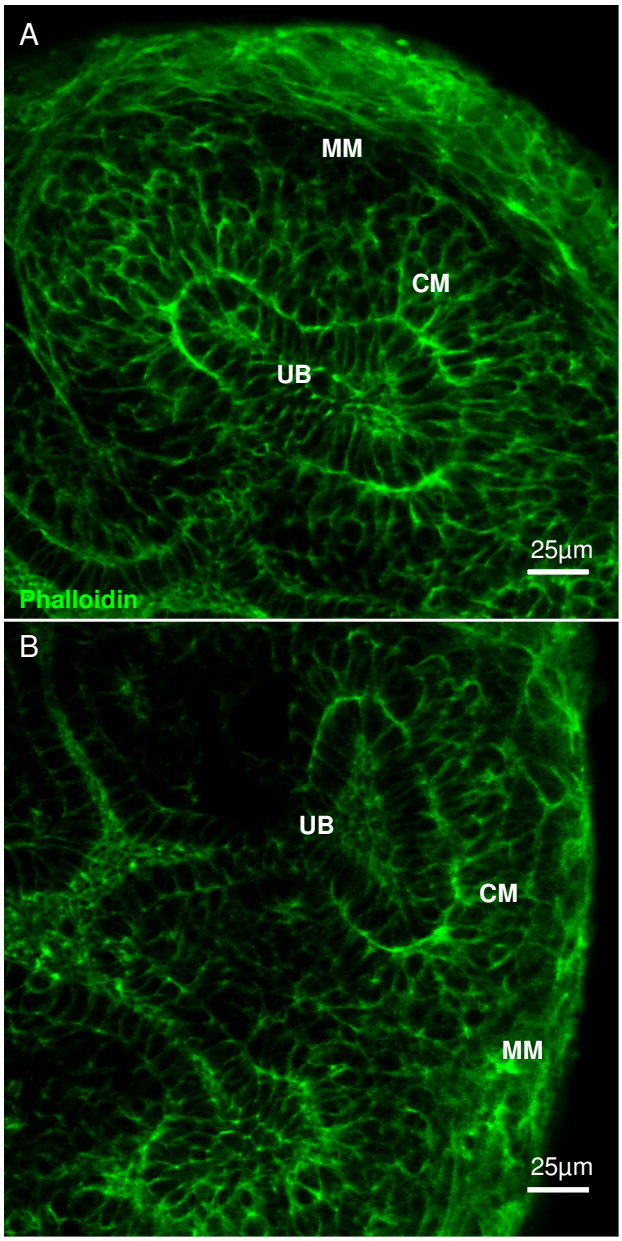


Fig.3.3 The effects of Rac1 inhibition filamentous actin distribution in the kidney

Fig.3.3 shows the effects of NSC23766 on the distribution of filamentous actin in the ureteric bud tips. (A) Kidney cultured for 72hrs in CKCM. (B) Kidney cultured for 72hrs in CKCM supplemented with 75µM NSC23766. Kidneys were stained for filamentous actin using FITC-conjugated Phalloidin. UB-ureteric bud, MM-metanephric mesenchyme, CM-cap mesenchyme.

3.2.4 Inhibition of Rac1-GTPase does not affect tip-to-stalk ureteric bud differentiation.

The ureteric bud can, during early kidney development, be divided into at least two discrete regions; stalks and tips. The stalk regions make up the majority of the arboreal collecting duct system, and the ureteric bud tip regions only defines the extreme terminal parts of the collecting duct system most distal to the Wolffian Duct (Michael, Sweeney, and Davies 2007). During early stages of ureteric bud growth, the cells in the ureteric bud tips are capable of differentiating in a tip-stalk direction (Shakya, Watanabe, and Costantini 2005; Marose et al 2008). Interestingly, stalk regions can also be made to revert to a tip-like state and regain the capability to induce nephron formation and to branch (Sweeney, Lindström, and Davies 2008). One potential explanation for the reduced branching and growth exhibited by kidneys cultured in the presence of Rac1 inhibitor NSC23766 could be a failure of the maintenance of the ureteric bud tip identity in appropriate areas.

I decided to investigate whether the stalk-domain was properly defined and excluded from the tip regions when Rac1 activity is inhibited. The lectin Dolichos biflorus agglutinin (DBA) binds to the ureteric bud stalk and is excluded from the ureteric bud tips (Michael, Sweeney, and Davies 2007). This exclusion is complementary to the gene Wnt11 which is normally expressed only in the ureteric bud tips (Kispert et al 1996; Michael, Sweeney, and Davies 2007). If ureteric bud branching is artificially inhibited by reducing GDNF signalling, the DBA binding expands to the tip regions (Michael, Sweeney, and Davies 2007). This suggests that DBA can be used as an indicator of whether the tip identity is maintained and functional. Kidney cultures were set up and cultured for 48hrs in control conditions or in medium with 75µM NSC23766. After fixation, the cultures were stained using anti-β-laminin which was detected with anti-rabbit-TRITC. FITC-conjugated DBA was also used. β-laminin is present in the whole of the ureteric bud. Controls showed a normal distribution of DBA (Fig.3.4A). DBA is clearly excluded from the tips. Demonstrating the areas of co-localisation of laminin and DBA-binding (Fig.3.4A') clearly shows that co-localisation does not occur in the ureteric bud tips. Cultures in Rac1 inhibitor displayed the same exclusion of DBA from the ureteric bud tips (Fig.3.4B) which is

again highlighted by illustrating the co-localisation of DBA-binding and laminin (Fig.3.4B'). These results indicate that normal ureteric bud stalk-tip identities are maintained. In order to ensure that the ureteric bud tip regions are normal, in addition to determining whether the ureter forms normally, it would be necessary to look at markers specific for those regions. Wnt11 would be a good marker to study the ureteric bud tip regions and Uroplakin, a marker of ureter differentiation, would be suitable to determine whether the ureter differentiates normally (Kispert et al 1996; Brenner-Anantharam et al 2007).

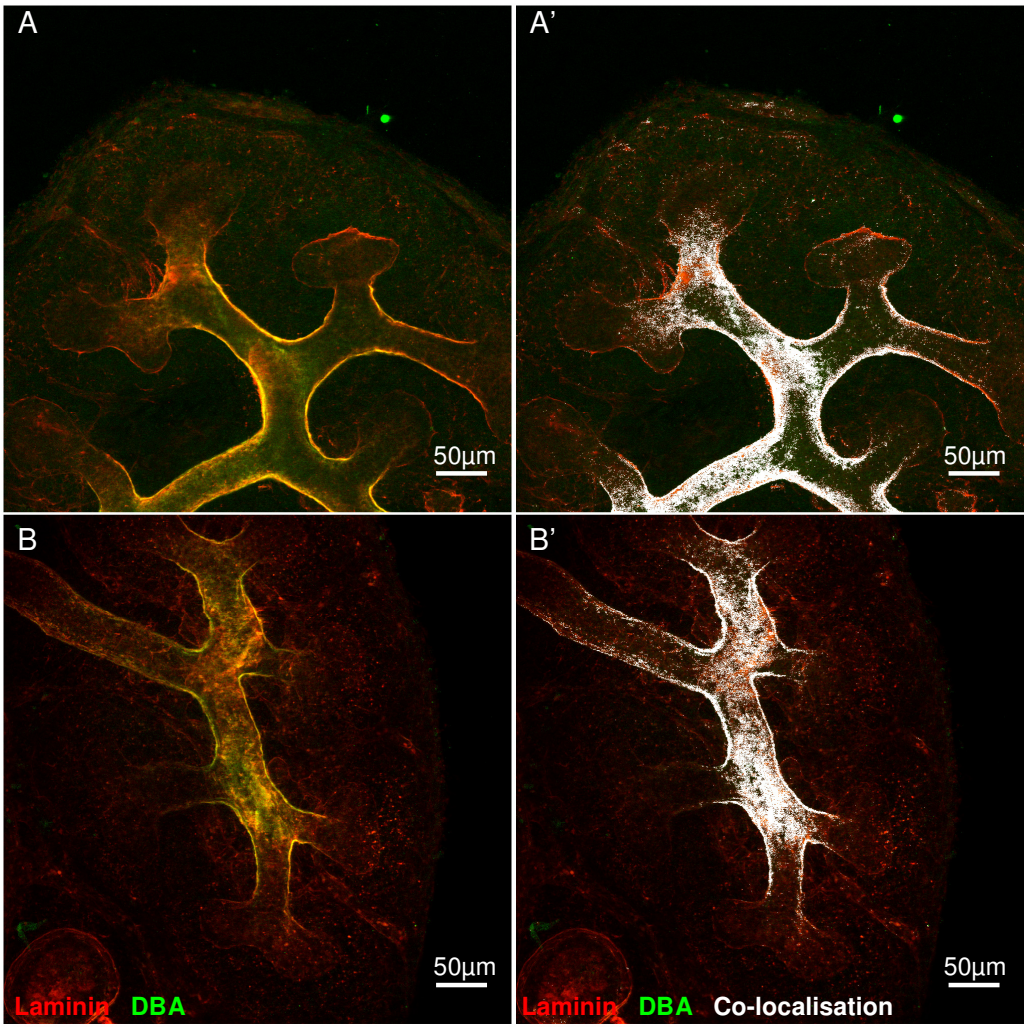


Fig.3.4 The effects of Rac1 inhibition on kidney stalk-tip boundaries

Fig.3.4 shows kidneys that were grown for 48hrs in CKCM with or without Rac1 inhibitor NSC23766 before being stained for laminin-rabbit and FITC-conjugated DBA-lectin. The β-laminin antibody was detected with anti-rabbit-TRITC. (A) Kidney cultured in CKCM. (B) Kidney cultured in CKCM with 75µM NSC23766. (A',B') shows the points of co-localisation of laminin and DBA-lectin demonstrating the absence of DBA binding from ureteric bud tips in both conditions.

3.2.5 Inhibition of Rac1-GTPase does not negatively affect nephrogenesis or nephron maturation and differentiation.

Kidney development is regulated by elaborate cross-talk between the ureteric bud and the metanephric mesenchyme components (Sainio et al 1997; Carroll et al 2005). Mesenchymal factors such as WT1 and GDNF have been shown to be necessary for ureteric bud induction and branching (Kreidberg et al 1993; Sainio et al 1997). Inhibition of Rac1 in cultured kidneys results in reduced ureteric bud branching and reduced kidney growth. A possible explanation for these reductions might be a disturbed development of the mesenchymal portion of the kidneys as this could lead to abnormal mesenchymal-to-ureteric bud signalling. Several reasons make it interesting to consider whether Rac1 could regulate nephron formation. Firstly, the branching defects observed upon Rac1 inhibition may not have been a result of direct effects of the inhibitor on the ureteric bud as demonstrated in Wnt4 mutant mice (Wnt4 is only expressed in the nephrogenic structures in the metanephric mesenchyme) where the absence of nephrons also results in greatly reduced branching (Stark et al 1994). Secondly, Rac1 is a component of the planar cell polarity pathway and it has previously been shown that Rac1 regulates renal progenitor cell cultures by controlling the culture size (Osafune et al 2006). In addition, the downstream effector of Rac1, JNK, regulates the differentiation and expression of markers for nephron maturation in progenitor cell cultures (Osafune et al 2006). Thirdly, Rac1 might regulate nephron development in a previously unknown mechanism. As discussed in Chapter 1, the mechanisms behind the formation of the pretubular aggregates, renal vesicles and glomerular cleft are unknown. One possibility is that Rac1 might control part of these processes, either by affecting cellular motility which could be important for pretubular aggregate formation or perhaps by the regulation of cell adhesion or the progression through the mesenchymal-to-epithelial transition. Regulation of Rac1 activity has been linked to the mesenchymal-to-epithelial transition during somitogenesis (Takahashi et al 2005).

Here, I have divided nephrogenesis into three parts where I investigate the important of Rac1. For a complete picture of nephron formation, please read the detailed

descriptions in Chapter 1. The first stage is the developmental phase of the nephron that spans the period between the initial induction of cap metanephric mesenchyme until the stage where the epithelial renal vesicle is formed. The second stage is the phase that encompasses the comma- and s-shaped body stages. These are particularly important as a large array of processes is initiated at this point. Most notably of these processes are the formation of the glomerular cleft, specification of the parietal and visceral epithelia, podocyte differentiation, and the proper structural and genetic differentiation of the proximal-distal axis (Saxen 1987). The third stage contains the development and maturation that occurs post s-shaped body stage. This is mainly characterised by the differentiation of the elements that contribute to the segmentation along the proximal-distal axis. Several cadherins display selective expression patterns along the proximal-distal axis suggesting differential adhesion might play a role during nephron maturation (Cho et al 1998; Dahl et al 2002).

3.2.5.1 Inhibition of Rac1-GTPase does not affect the cap mesenchyme or the formation of renal vesicles.

The first nephron stages to be considered here are the cap mesenchymes, the pretubular aggregates and the renal vesicles that are the earliest nephrogenic structures (Little et al 2007). The cap mesenchyme surrounding the ureteric bud tips expresses neural cell adhesion molecule (NCAM) and this expression endures during the formation of the nephrogenic pre-tubular aggregates and into the renal vesicle stage (Klein et al 1988a; Bard et al 2001). This makes it an excellent marker to visualise early nephron formation from the stage of cap mesenchyme until renal vesicle.

Kidney cultures were set up in control conditions (Fig.3.5A) or in 75µM NSC23766 (Fig.3.5B) for 72hrs and stained for NCAM and counterstained with TO-PRO-3 to visualise the nuclei. Confocal microscopy revealed NCAM distribution in controls and in experimental set-ups with indistinguishable localisation patterns. The cap mesenchyme in controls and in experimental conditions displayed similar levels of NCAM in the cap mesenchyme. The cap mesenchyme is highly ordered as is also

shown by immunofluourescent imaging of its actin cytoskeleton (Fig.3.3). Cultures with Rac1 inhibitor also displayed morphologically normal renal vesicles compared to controls. This data strongly suggests that inhibition of Rac1 does not perturb normal renal vesicle formation. The renal vesicles in control conditions and in experimental conditions appeared similar in size. The size of the nephrons was not quantified. The reason for this is that it would be very difficult to generate meaningful data regarding the nephron sizes. This is because nephrons form in a sequential and continuous manner and an average size for a nephron would thus have to depend on its exact stage. Whilst it is possible to stage a nephron with some certainty, even nephrons within a stage will vary in size depending on whether they are in the beginning of that stage or just about to progress into the next developmental phase. The size of the nephrons could be an important issue since it has been shown that the size of nephron progenitor cell cultures is regulated by JNKs (Osafune et al 2006).

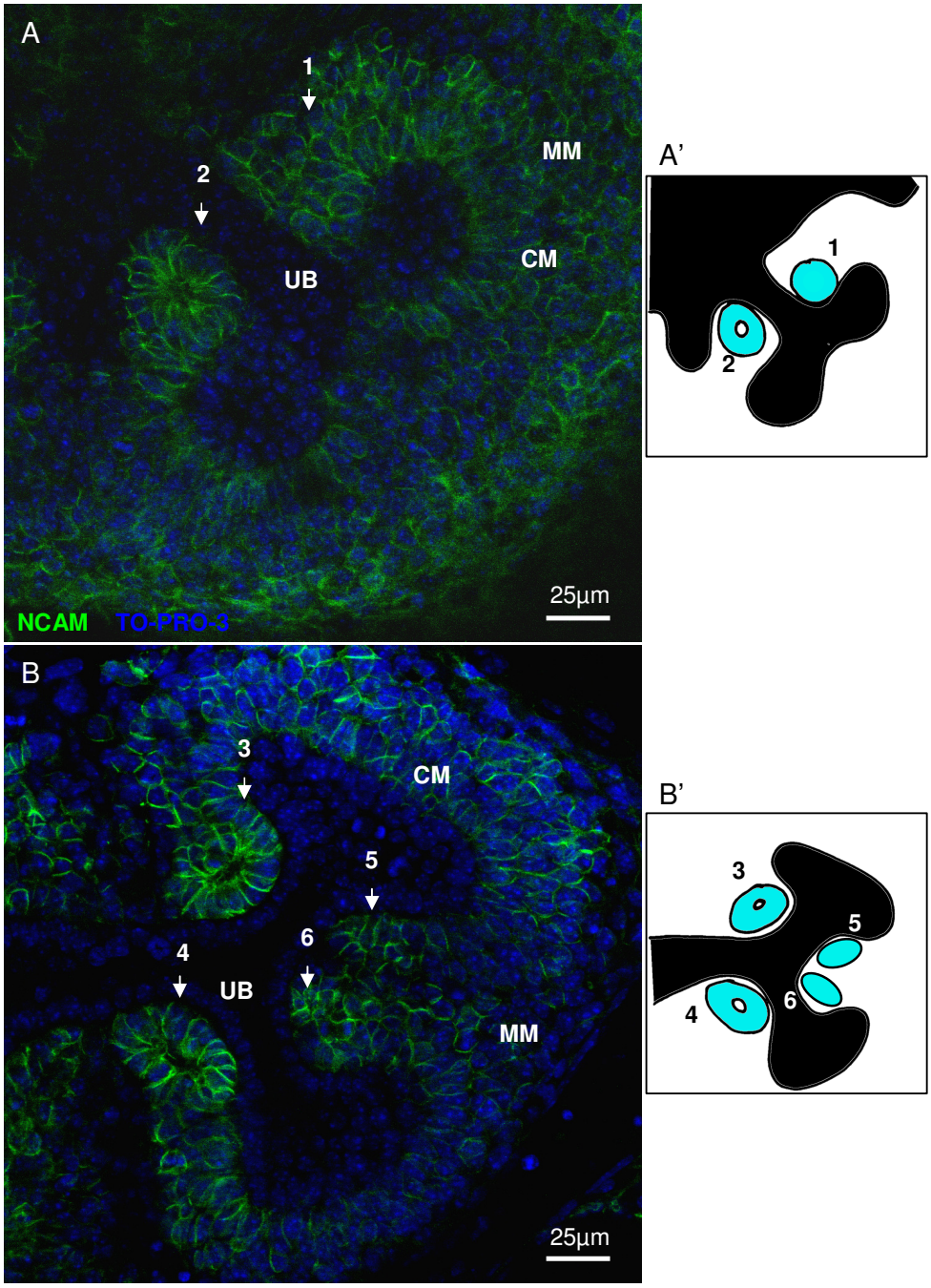


Fig.3.5 The effects of Rac1 inhibition on early nephrogenesis.

Fig.3.5 shows E11.5 kidneys that were cultured in CKCM with or without 75µM Rac1- inhibitor NSC23766 for 72hrs and fixed in -20ºC methanol before being stained with anti-NCAM. The NCAM antibody was detected using anti-mouse-FITC. TOPRO-3 (Cy5) was used to stain the cell nuclei. (A) Kidney cultured in CKCM. (B) Kidney cultured in CKCM with 75µM NSC23766. NCAM expression appears normal and pretubular aggregates and renal vesicles are formed normally in both culture

conditions. White arrows – pretubular aggregates and renal vesicles. (A' and B') show schematic representations of numbered nephrons in (A and B). Diagrams were produced by careful inspection of nephrons at maximum resolution. UB- ureteric bud, MM-metanephric mesenchyme, CM-cap mesenchyme.

3.2.5.2 Inhibition of Rac1-GTPase does not affect the formation of comma- and s-shaped bodies or podocyte differentiation.

Subsequent to the renal vesicle stage is the comma-shaped body stage and then the s-shaped body stage (Saxen 1987; Little et al 2007). In the mouse, Wilms Tumour 1 (WT1) is expressed at low levels in the metanephric mesenchyme and, as the nephron matures and develops, the expression of WT1 become significantly elevated in the comma-shaped body and in the S-shape body (Armstrong et al 1993; Huber et al 2000). Comma- and s-shaped body stage nephrons are characterised by the differentiation of the parietal and visceral epithelia. Here I use WT1 as a tool to indicate both podocyte differentiation and the development of the visceral and parietal epithelia. WT1 is a suitable marker for this because it can be use to both show that podocyte differentiation initiates and it can also be used as a structural marker because its localisation pattern highlights the characteristic physical appearance of the flattened parietal epithelium which is separated from the columnar visceral epithelium by the Bowman's space (see Fig.1.2 for a structurally labelled nephron).

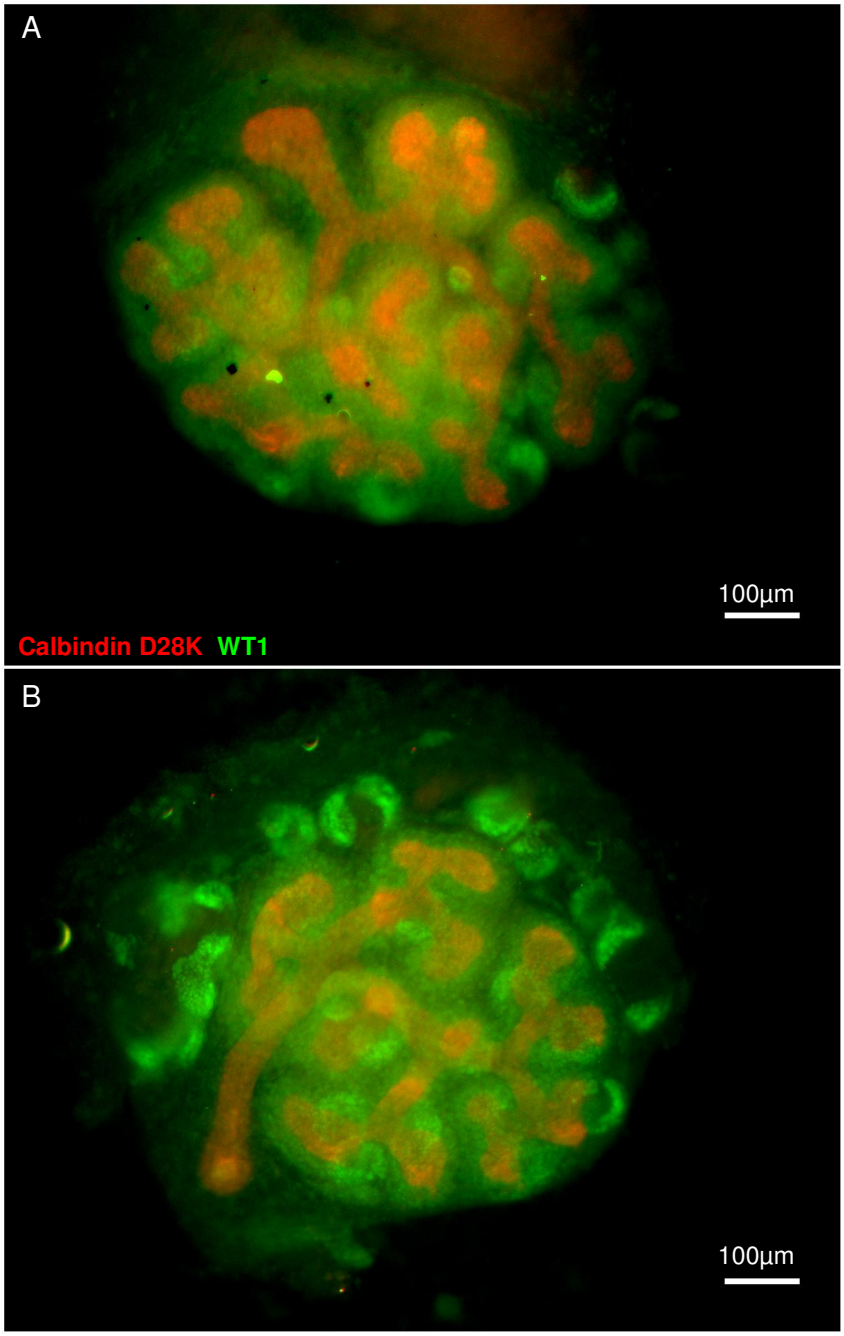
Kidney cultures and spinal cord-induced mesenchyme cultures were set up to investigate whether Rac1 plays a role during the formation and differentiation that takes place to form the comma- and s-shaped bodies. The purpose of using both sets of cultures was to:

A) In whole kidney cultures demonstrate whether podocyte differentiation and comma-, s-shaped body stage nephron formation is affected by Rac1 inhibition.

B) Assess whether any potential effects of Rac1 inhibition were a result of Rac1 affecting the ureteric bud.

As described in Chapter 2, metanephric mesenchyme can be separated from the ureteric bud at E11.5 and cultured in the presence of spinal cord in order to induce nephron formation (Saxen 1987). In such cultures, typical comma- and s-shaped bodies form (Saxen 1987). Whole kidney cultures were set up in control conditions

(Fig.3.6A) or in 75µM NSC23766 (Fig.3.6B). Cultures were maintained for 72hrs and subsequently fixed and stained for Calbindin D-28K and WT1. Calbindin D-28K is found exclusively within ureteric bud during early kidney development. Nephrons that formed in the presence of Rac1 inhibitor NSC23766 showed similar development to those nephrons that formed in control cultures. Nephrons in both cultures had progressed to a similar degree of maturation. Podocyte differentiation occurred in both conditions. Spinal cord induction cultures in control conditions (Fig.3.6C) and in experimental conditions (Fig.3.6D) showed normal nephron development and podocyte differentiation. Control and experimental cultures displayed structurally correct visceral and parietal epithelia separated by a lumen. The spinal cord induction cultures were also stained for Calbindin D-28K to ensure that no ureteric bud fragments remained in the cultures. The TRITC stain in the images is shown to display the fluorescence background levels. These results indicate that inhibition of Rac1 does not disturb nephron development from renal vesicle stage to comma- and s-shaped body stages in terms of visceral and parietal epithelia formation. Nor does it affect normal initiation of podocyte differentiation.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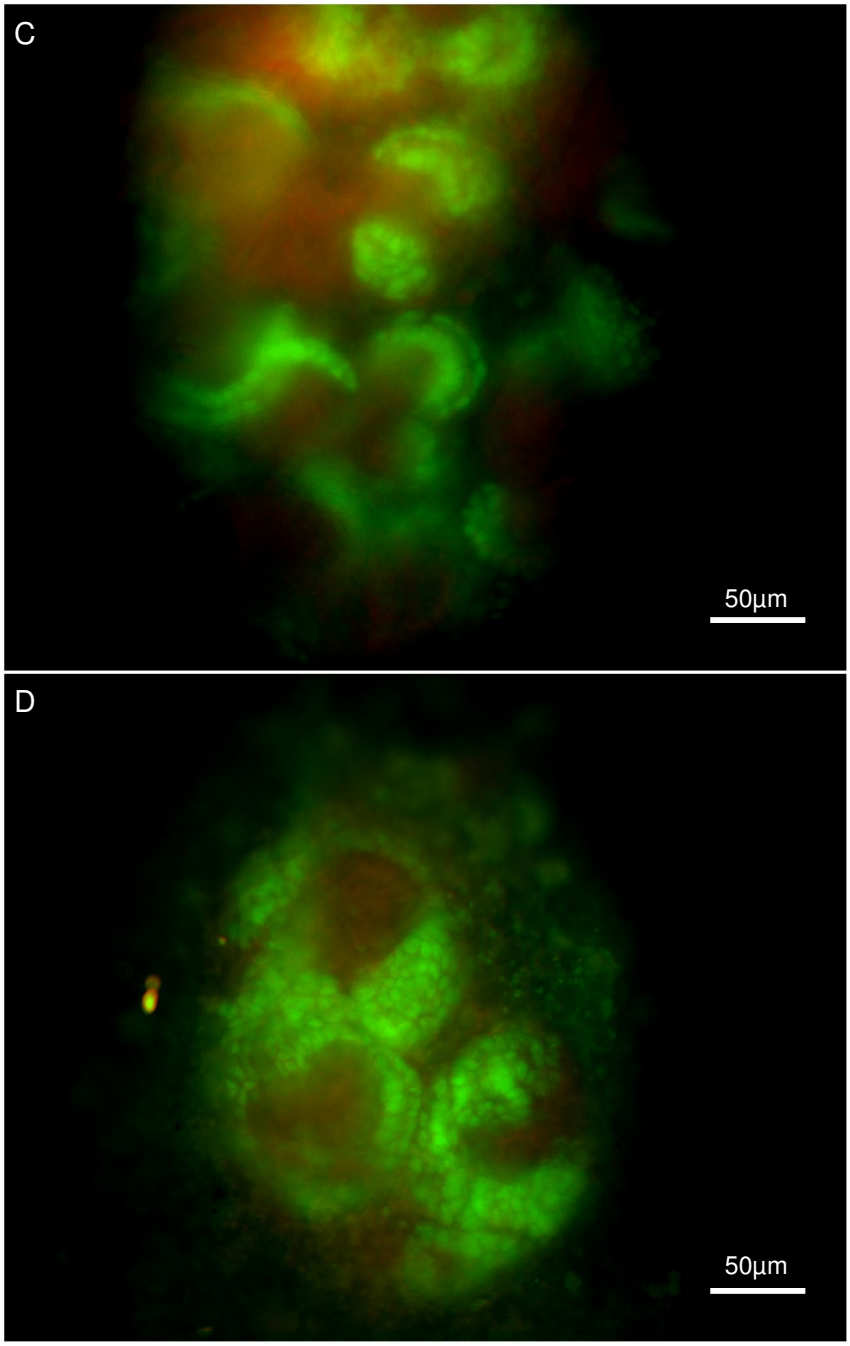


Fig.3.6 The effects of Rac1 inhibition on the formation of comma-shaped and s-shaped nephrons and podocyte differentiation

Fig.3.6 shows whole kidney cultures (A,B) and metanephric mesenchyme that was induced using spinal cord to form nephrons (C,D) in the presence or absence of 75µM NSC23766. The cultures were

stained with Calbindin D-28K-Rabbit and WT1-Mouse antibody stains. Primary antibodies were detected using anti-rabbit-TRITC and anti-mouse-FITC. (A) Whole kidney in CKCM. (B) Whole kidney in CKCM supplemented with 75μM NSC23766. (C) Metanephric mesenchyme in CKCM. (D) Metanephric mesenchyme in CKCM supplemented with 75 μM NSC23766 medium.

3.2.5.3 Inhibition of Rac1-GTPase does not affect nephron maturation.

Rac1 inhibition appears not to be important during early stages of nephron formation, or during the formation of podocytes and the glomerular cleft, as shown in previous sections. The final stage that I decided to investigate follows the s-shape body stage. This stage of nephron development is mainly characterised by the nephron elongating and acquiring the segmentation that is associated with the mature nephron as well as becoming vascularised (Saxen 1987). During the formation of the proximal-distal axis, a planar axis, the nephron organises itself into several molecularly distinct segments. The mature nephron has recently been characterised in terms of the expression pattern of the large number of solute-carriers that are found in the nephron. The mature murine nephron displays, 8 solute-carrier defined segments (Raciti et al 2008). These segment markers, in addition to others, can be used as indicators for nephron development as many are only expressed when the nephron matures. I set up experiments in order to evaluate whether the inhibition of Rac1 affects proximal-distal axis differentiation.

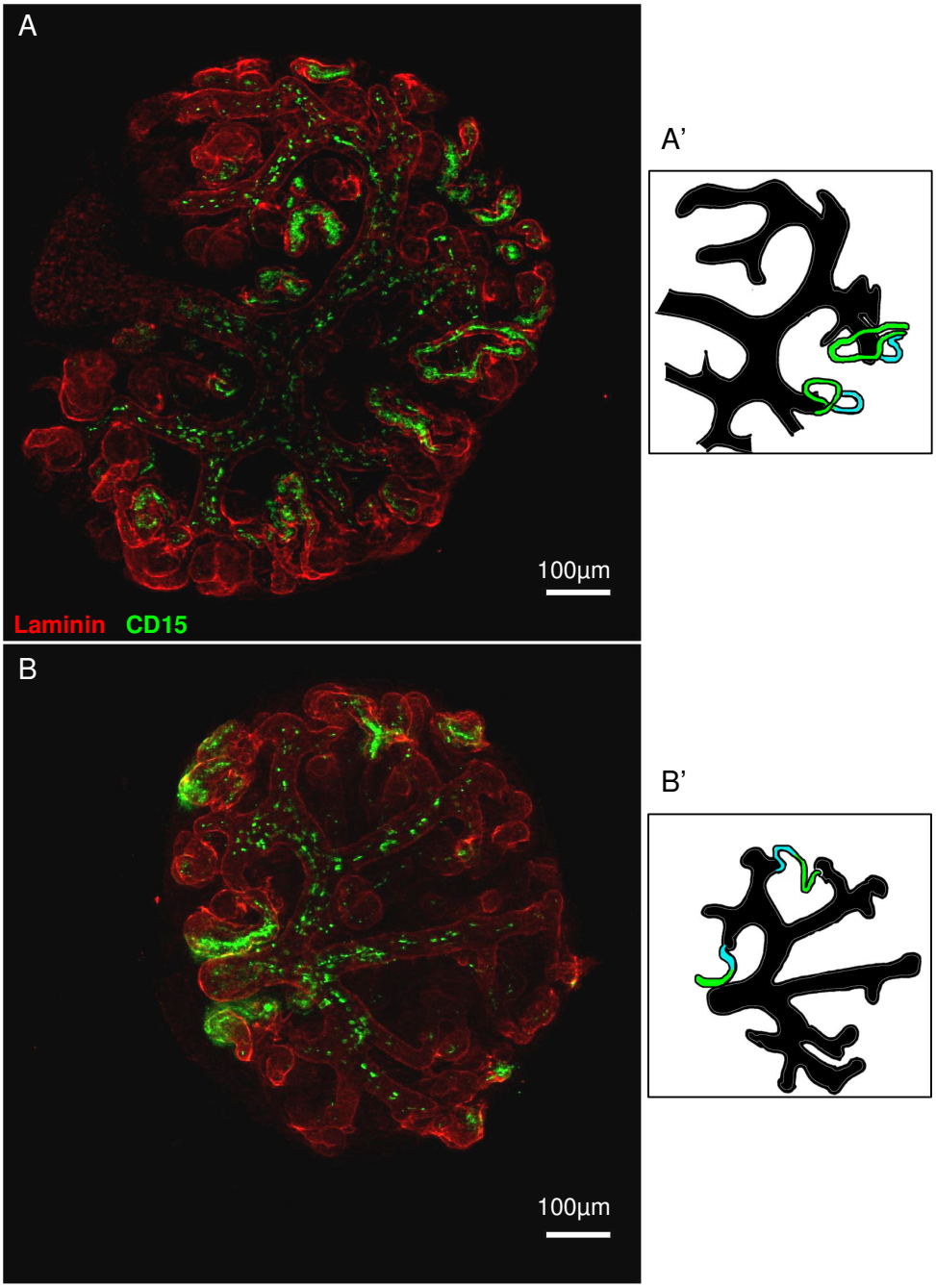
CD15 is a protein marker exclusively present in the proximal portions of the developing tubule (Bard and Ross 1991; Davies 1994). Expression of CD15 can only be weakly detected at the s-shaped body stage but becomes higher during maturation. Kidney cultures were set up in control conditions (Fig.3.7A,C) or in medium with 75μM NSC23766 (Fig.3.7B,D). The cultures were maintained for 144hrs to allow for progression beyond the s-shaped body stage. The cultures were fixed and stained for CD15 and β-laminin to show the proximal tubule and the basal membrane. Nephrons forming in control conditions and those forming in experimental conditions showed normal morphology and displayed typical localisation of CD15 to the apical domain of proximal tubules. The nephrons also showed fusion to the collecting duct system. The segments of the nephrons adjacent to the ureteric bud (distal tubule) were negative for CD15 staining. It has previously been shown that JNK, a downstream effector of Rac1 in the planar cell polarity pathway, is necessary for the expression of markers indicative of nephron maturation in nephron progenitor cell cultures (Osafune et al 2006). As previously mentioned, the nephron progenitor cells cultures

were derived from single-cell colonies of Sall1 positive cells that were expanded and cultured on Wnt4 expressing 3T3 cells (Osafune et al 2006). This makes those cultures very different to the whole kidney cultures I have used here since the nephron progenitor cultures are two dimensional and at the very best only contain parts of the environment that nephrons in a kidney are normally exposed to. I found no evidence to support the hypothesis that Rac1 is necessary for nephron maturation as the maturing nephrons that formed during Rac1 inhibition were morphologically normal and displayed characteristic proximal-distal differentiation.

As a summary to the data presented here, which relates to nephron formation and development, this data suggests that nephron development, as has been studied at several different stages of maturity, appear to proceed normally in the presence of 75μM of the Rac1 inhibitor drug NSC23766. This apparently normal nephron development makes it unlikely that the effects exhibited by the inhibitor on branching and growth rates were secondary effects resulting from abnormal nephrogenesis. The same concentration of inhibitor produced the branching and growth defects. This is one of the areas that unfortunately do not display the depth of investigation that I would have wished for but this was suffered in order to focus on the Rho-kinase research. This data does not contradict the work by Osafune and colleagues (Osafune et al 2006), however, it does not directly agree either. Their research showed that inhibiting JNK1 and JNK2 can alter the 'differentiation' of the nephron progenitor cell cultures by blocking the expression of several genes that indicate nephron maturation. The reason for considering that the inhibition of Rac1 might result in similar results is based on the knowledge that Rac1 regulates JNK in the planar cell polarity pathway (Habas, Dawid, and He 2003), a pathway thought to be active in the nephrons (Park, Valerius, and McMahon 2007). A real possibility is that, although the concentration of Rac1 inhibitor used here produces branching defects and correlates well with what others have used (Ewald et al 2008), I have inhibited Rac1 insufficiently to alter nephrogenesis. The explanation might lie in that although I might have inhibited Rac1 activity by 80%, nephron formation might only require 5% of normal Rac1 activity. This was the next step that would have been pursued if the Rac1 research had been prioritised. It is possible to assess the level of

Rac1 activity utilising an assay system where active Rac1 bound to GTP is pulled down using a pull-down assay binding active Rac1 to a PAK1 fusion protein tagged to glutathione. Thus it might have been possible to affect nephron formation with more optimised levels of Rac1 inhibition.

It is important to bear in mind that Rac1 might not act via the planar cell polarity pathway in the nephrons and therefore the effects of JNK1 or JNK2 inhibition are completely unrelated. Equally possible is that JNK1 and JNK2 are not regulated via the planar cell polarity pathway but are activated through different signals. It is also important to note that the effects of JNK1 and JNK2 inhibition were studied in a more artificial environment than the whole kidney cultures used here which are likely to be significantly better at mimicking in vivo development. Another prospect is that Rac1 does not actually play a role during nephron development, yet still negatively affect the transduction of signals from the nephron to surrounding tissues and in that way regulate ureteric bud branching and growth. Rac1 might play a role in other components of the metanephric mesenchyme, such as uninduced mesenchyme or the stroma, which have not been investigated here.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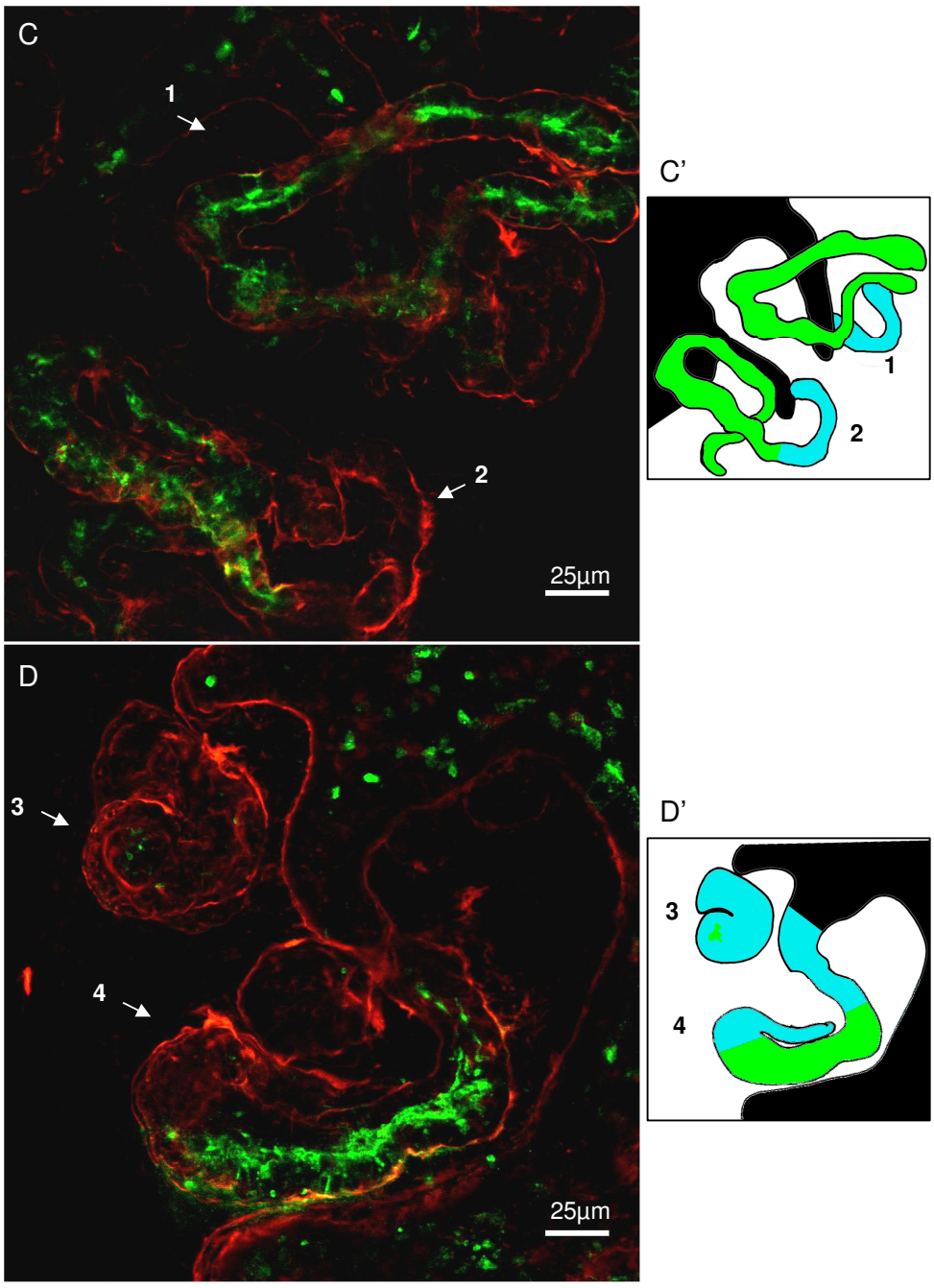


Fig.3.7 The effects of Rac1 inhibition on nephron proximal-distal segments

Fig.3.7 shows whole kidney cultures set up in CKCM with or without 75µM Rac1- inhibitor NSC23766 and cultured for 144hrs. Cultures were fixed in -20ºC methanol before being stained for mouse-β-laminin to mark the basement membrane and mouse-CD15 which is specific for proximal

tubules. The primary antibodies were detected using anti-rabbit-TRITC and anti-mouse-FITC. (A,C) Kidneys cultured in CKCM. (B,D) Kidneys cultured in CKCM with 75µM NSC23766. White arrows – nephrons expressing CD15 normally. (A'-D') show schematic representations of numbered nephrons in (A-D). Nephrons are in turquoise and the UB in black. Green portions of the nephron signifies regions positive for CD15. Diagrams were produced by careful inspection of nephrons at maximum resolution.

3.2.6 Proliferation as a mechanism for growth and branching in the kidney.

Previous experiments discussed in Chapter 3 have shown that inhibition of Rac1 negatively affects the rate of branching and growth in kidneys in culture and that these effects are likely not to be a result of abnormal nephron development. As is described in Chapter 1, cellular proliferation is a potential mechanism of morphogenesis that may be required not only for ureteric bud induction but also during subsequent branching and growth (Michael and Davies 2004). This was demonstrated by both the relatively higher rates of cellular proliferation seen in the ureteric bud tips, but also by the evidence showing that inhibition of proliferation results in reduced ureteric bud induction (Michael and Davies 2004). One example of a proliferation stimulating factor is glial cell line derived growth factor (GDNF), which is considered a central kidney growth factor (Michael and Davies 2004; Shakya, Watanabe, and Costantini 2005). Rac1 has been demonstrated to play an important role during cell cycling and proliferation as is covered in the introduction to Chapter 3. In light of previous experiments in Chapter 3, and from the knowledge that Rac1 can regulate proliferation rates (Minden et al 1995), I investigated the possibility that the effects that inhibiting Rac1 had on branching and growth were a result of reduced proliferation. This is a prospect that would fit well with the unchanged morphology of the kidney and the ureteric bud as well as the overall "general reduction" in kidney growth that is displayed in cultures with Rac1 inhibitor. The possibility that Rac1 might regulate proliferation was always considered and experimental analysis of this was initiated directly when it was demonstrated that the inhibition Rac1 reduces branching and overall growth. These experiments represent an important portion of the findings presented in this chapter. The original thought was to discuss the effects of Rac1 on proliferation directly after I discussed how Rac1 reduces branching and growth. However, because several experiments were carried out, each adding support to the previous ones, it became clearer to discuss all experiments together. Thus I have decided to aggregate these findings and discuss them in a more unified and coherent manner with the advantage of looking back at the previously described result and perhaps offer some more insightful conclusions. The experiments are presented in sections 3.2.6-3.2.8. In

these sections I will discuss: (1) experiments studying proliferation in the ureteric bud tips using Bromodeoxyuridine; (2) problems with the approach using Bromodeoxyuridine and the solutions to these problems using phosphorylated histone 3 as a mitosis marker; (3) possibilities of mimicking branching defects by inhibiting proliferation; (4) the prospect of proliferation directly regulating branching as well as Rac1 only regulating proliferation in the kidney.

3.2.7 Inhibition of Rac1-GTPase negatively affects proliferation in the kidney as shown using BrdU.

Cell proliferation was analysed using Bromodeoxyuridine (BrdU) in a manner previously optimised by Michael and Davies and used for publication (Michael and Davies 2004). A detailed protocol for BrdU experiments and the image analysis carried out can be found in Chapter 2. Kidneys were cultured for 46hrs of which the last 16hrs were in 100µM BrdU. Cultures were maintained in either control conditions or in 75µM NSC23766. After fixation, the cultures were staining for BrdU and Calbindin D-28K. Kidneys were scanned on a Leica TCS-NT confocal microscope at 3µm intervals on the z-axis. BrdU incoproration was expressed as the average number of BrdU positive nuclei per 1000µm^2 of kidney tissue as discussed by Michael and Davies (Michael and Davies 2004). BrdU positive nuclei were identified by applying a consistent threshold to reduce background and identify true positives. The choice to describe proliferation of cells based on the area, rather than as a percentage of the number of nuclei, stemmed from the paper by Michael and Davies (Michael and Davies 2004). They discuss that the thickness of the kidneys in culture makes it difficult to reliably label nuclei. Michael and Davies also mention that using histological sections is not ideal either since it can be difficult to differentiate ureteric bud tips from other tubules in these sections (Michael and Davies 2004).

BrdU positive nucleu were counted in the ureteric bud tips, as described by Michael and Davies (Michael and Davies 2004). The average number of BrdU positive nuclei

per 1000μm^2 was found in ureteric bud tips of kidneys cultured in control conditions (Fig.3.8A) and those in experimental cultures (Fig.3.8B). Two experiments were carried out. In the first experiment, 16 and 9 ureteric bud tips from kidneys in control and experimental conditions were analysed. In the second experiment, 11 and 9 ureteric bud tips were analysed, respectively. The difference in the number of tips analysed stems from that the kidneys in Rac1 inhibitor conditions branched less. Data from experiment 1 suggest an average decrease of 24% of BrdU positive nuclei in ureteric bud tips in Rac1 inhibitor conditions, p=0.066 as was analysed using an unpaired one-tailed Student's t-tests (Fig.3.8C). The variability is expressed using the standard error of the mean (SEM). Data from experiment 2 displayed an average decrease of 24%, p=0.0027 (Fig.3.8C). Combining the data from experiment 1 and experiment 2 for analysis (27 control ureteric bud tips and 18 experimental tips) resulted in the data showing an average of 20% reduction in the number of BrdU positive nuclei in experimental ureteric bud tips, p=0.054 (Fig.3.8C). A reduction of BrdU incorporation, and thus also possibly proliferation rates, could be hypothesised to lead to a reduction in the ureteric bud size. In order to determine whether this was the case, the data from section 3.2.1 was reanalysed. The branch lengths were measured in every kidney from the first bifurcation of the ureteric bud and of along each branch to every ureteric bud tip. This gave a good estimate to what degree if any, that the ureteric bud had decreased in size. For the kidneys in control conditions, 5 kidneys were analysed and 154 branch distances were measured. For the kidneys in 75μM NSC23766, 6 kidneys were analysed and 120 branch distances were measured. Again, the lower number of branch distances was a result of the experimental kidneys displaying reduced branching. The average branch length for control kidneys was 465.7μm ± 7.6μm (SEM) and for experimental kidneys it was 343.9μm ± 7.0μm (SEM). The average branch lengths in kidneys cultured in 75μM Rac1 inhibitor was 73% of that in control kidneys ($p=1.72x10^{-25}$) as analysed using an unpaired one-tailed Student's t-test. This reduction in ureteric bud length closely correlates with the reduction in BrdU incorporation seen at the same concentration of Rac1 inhibition.

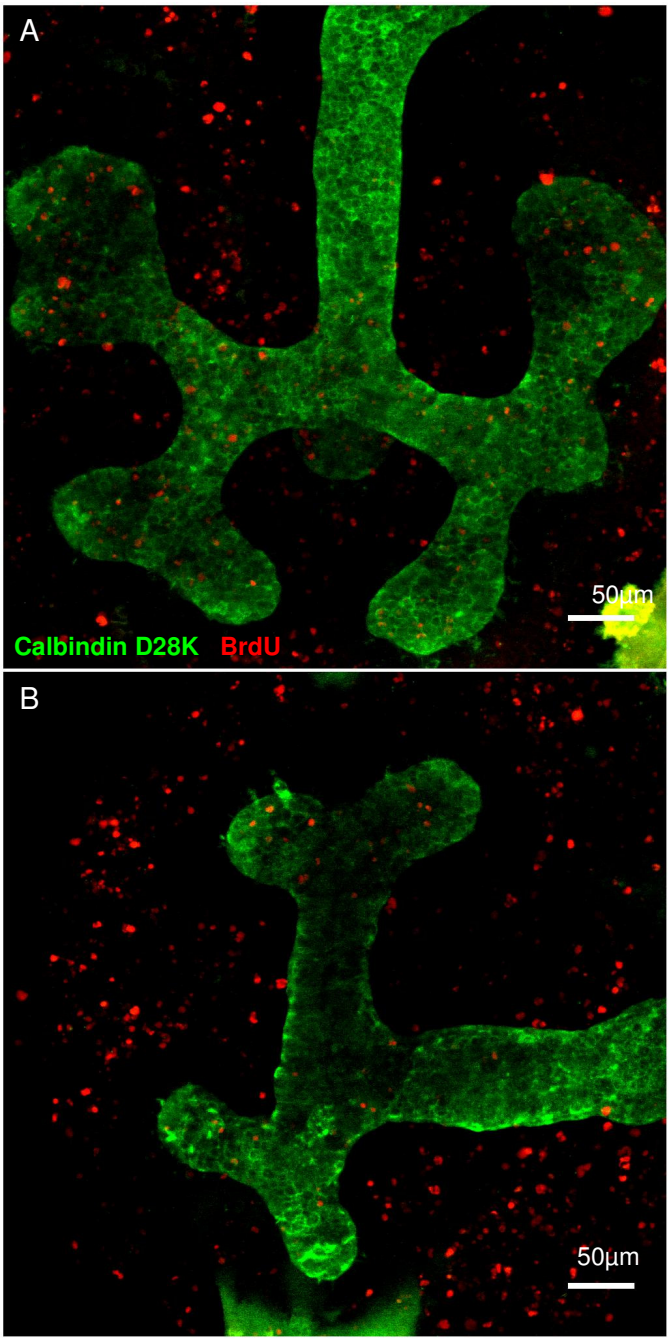
The BrdU analysis provided some problems that were necessary to tackle. The problems and the solutions that were chosen are discussed in the following paragraph. I have chosen to discuss these issues here because it is important to understand the problems with BrdU and the reasons for changing the strategy of indicating cellular proliferation before continuing with the next results sections.

The data from the BrdU analysis were statistically significant; however, the samples displayed a large degree of variation in terms of the average number of BrdU positive nuclei per 1000μm^2. For the controls, the average number of BrdU positive nuclei per 1000μm^2 was 4.14 in experiment 1 and 7.7 positive in experiment 2. This is almost a 2 fold difference. This variation might be accountable for by the problems with BrdU antibody staining, which on kidney explants proved to be difficult and often yielded very variable background noise from non-specific antibody binding. A second problem was the presentation of BrdU positive nucleu as a fraction based on a unit area. Combining the measurement of a specific number of nuclei with an area presented problems mainly because of the possibility of variable cell densities. Although this was not a major problem when analysing proliferation in the ureteric bud, which is relatively uniform, it did pose problems when considering extending the analysis to the rest of the kidney. The metanephric mesenchyme might have displayed large variations in cell density in particular as a result of nephron formation thus making it difficult to interpret any results in a meaningful way. A third problem might be a result of analysing only ureteric bud tips and not whole sections. The analysis of a smaller number of cells will automatically increase the potential for variation. Fourthly, one important aspect of using BrdU is the use of a suitable BrdU incubation period. This period had previously been described by Michael and Davies and it had been shown that the period could be reduced from 16hrs to 4hrs without reducing the proportional differences seen in BrdU incorporation displayed between ureteric bud tips and stalk regions (Michael and Davies 2004). This suggests that, as long as the incubation period was consistent between control and experimental conditions, any variation should be detectable. This section outlines the approaches taken in order to solve the problems described above.

The problems with BrdU originated mainly from that the BrdU antibodies did not work well. I chose to approach this problem by testing the use alternative markers of proliferation. Mitosis-specific marker, phosphorylated histone 3-Ser10 (H3(p)), which can be detected using a specific antibody, presented such an alternative. Using H3(p) as a marker for proliferation added the benefit of marking only those cells that were currently in mitosis, as opposed to BrdU which marked all cells that went through synthesis within the BrdU incubation period. As a result, using H3(p) eliminated both the use of BrdU and the necessity to determine an optimal BrdU incubation period. During the optimisation of the H3(p) strategy some issue arose. Firstly, problems were seen with the depth that the H3(p) would enter the kidney tissue and remain easily detectable with the secondary fluoropore-conjugated antibody. This could present problems during the quantification of H3(p) positive nuclei because poor antibody penetration could mimic low detection levels and falsely indicate a low proportion of cells in mitosis. In order to ensure that this would not affect the quantification, the sections analysed were consistently chosen so that the same µm depth from the surface of the kidney was analysed for each kidney and in each culture condition. Secondly, the H3(p) antibody displayed some diffuse non-specific binding which decreased the reliability of categorising nuclei as positive or negative for H3(p) binding. Antibody optimisation using blocking solutions with serum markedly reduced this. These modifications to the protocol appeared to provide good solutions since the antibody staining was thereafter consistent, clear and readily quantifiable in the sections analysed. The protocol is described in detail in Chapter 2.

Another problem with the BrdU technique was the issue that came from presenting the BrdU incorporation as a function of unit area, in particular as I wished to analyse proliferation throughout the kidney tissues. Michael and Davies opted for the ‘area’ approach because they described difficulties with reliably labelling nuclei in whole kidneys (Michael and Davies 2004). I encountered the same problems in attempting to label whole kidneys with nuclei marker TO-PRO-3. However, the penetration of the TO-PRO-3 nucleic dye was actually better than expected, and did not present

major problems when analysing sections that were all at the same µm depth, as described in the previous paragraph. Using the nucleic marker TO-PRO-3 allowed for rapid semi-automated quantification of the total number of nuclei. This meant that the number of mitotic cells could be expressed as the number of H3(p) positive nuclei over the total number of nuclei within a section. As a result it provided a robust protocol for measuring the mitotic index that could be used to analyse whole sections of kidneys as opposed to small regions. A detailed protocol is described in Chapter 2. Perhaps the best alternative is to utilise both approaches, BrdU and H3(p).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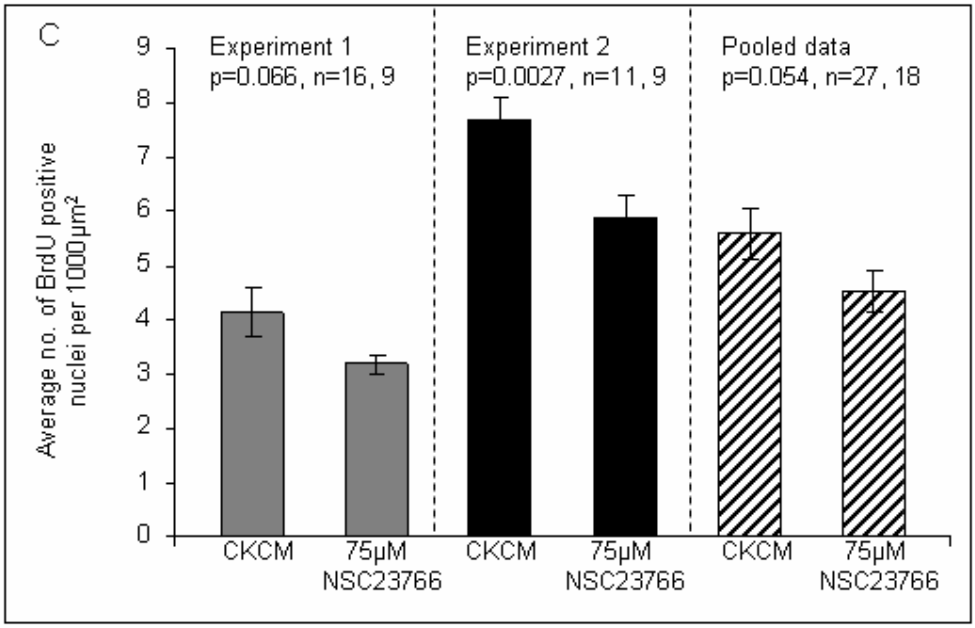


Fig.3.8 The effects of Rac1 inhibition on ureteric bud tip BrdU incorporation.

Fig.3.8 shows kidneys that were dissected and cultured in CKCM or CKCM supplemented with 75µM NSC23766. The kidneys were cultured for 30hrs and then exposed to 100µM BrdU for an additional 16hr period after which the kidneys were fixed and stained with rabbit anti-Calbindin antibody and mouse anti-BrdU antibody which were detected using anti-rabbit-FITC and anti-mouse-TRITC secondary antibodies. (A) Kidneys cultured in CKCM. (B) Kidneys cultured in CKCM supplemented with 75µM NSC23766. (C) Bar-Chart indicating the mean values ± SEM of BrdU positive nuclei detected per 1000µm^2 of ureteric bud tip for each treatment. P-values were derived separately using unpaired one-tailed Student's t-tests.

3.2.8 Mimicking effects of Rac1 inhibition by direct inhibition of proliferation.

The inhibition of Rac1 produced replicable effects on BrdU incorporation. It is important to verify these results using the more reliable H3(p) method. It is also necessary to determine whether the effects of inhibiting Rac1 on branching are entirely the result of the effects seen on BrdU incorporation and thus DNA synthesis. To resolve this, proliferation was directly inhibited using Methotrexate (MTX), which stops proliferation by blocking DNA synthesis and cell cycling (Chabner and Young 1973). The aim of directly blocking cell cycling, was to determine three things: (A) to show whether the direct inhibition of proliferation reduces the rate of branching; (B) to optimise MTX concentrations to inhibit branching to a degree equivalent to that achieved by Rac1 inhibition; (C) to determine whether the equally reduced branching as produced by MTX and Rac1 inhibitor results in an equivalent decrease in proliferation; as estimated by BrdU incorporation. The third point is the most significant as it will establish whether inhibition of Rac1 might affect other kidney functions in addition to cellular proliferation.

3.2.8.1 Direct inhibition of proliferation reduces branching.

In order to show whether direct inhibition of proliferation results in reduced branching, kidneys were cultured in control conditions or in a range of different MTX concentrations using the 500nM concentration previously used by (Davies et al 1995) as a starting point. Kidneys were cultured for 96hrs in control conditions, 50nM, 250nM or 500nM MTX (Fig.3.9A-D), respectively. Cultures were stained for Calbindin D-28K in order to allow for the quantification of branching by counting ureteric bud tips. Compared to kidneys cultured in control conditions (Fig.3.9A,E) (n=5kidneys), those cultured in 50nM MTX (Fig.3.9B,E) showed no decrease in the average number of branches or abnormal branching morphology ($p=0.44$, n=4 kidneys). P-values were derived using unpaired one-tailed Student's t-tests. Kidneys cultured in 250nM MTX (Fig.3.9C,E) exhibited a 32% reduction in branching ($p=0.08$, n=5 kidneys). Kidneys cultured in 500nM MTX (Fig.3.9D,E) displayed a

52% reduction in branching (p=0.002, n=5 kidneys). The branching morphology of the kidneys cultured in 250nM and 500nM MTX was different from that observed in control kidneys. It is also different from kidneys cultured in 75μM or 100μM NSC23766 (Fig.3.1C,D), where the degree of reduction in branching is similar. Although it was difficult to articulate the difference in branching morphology, the term "umbrella-like" was ascribed to those ureteric buds seen in high concentrations of MTX.

Firstly, these results demonstrate that the direct inhibition of proliferation results in decreased branching. Secondly, this data shows that 250nM MTX results in a decrease in branching (32%) that falls within the range also seen in cultures with 75μM of Rac1 inhibitor NSC23766 (25-43% decrease) (Fig.3.1C,E & Fig.3.2B,E).

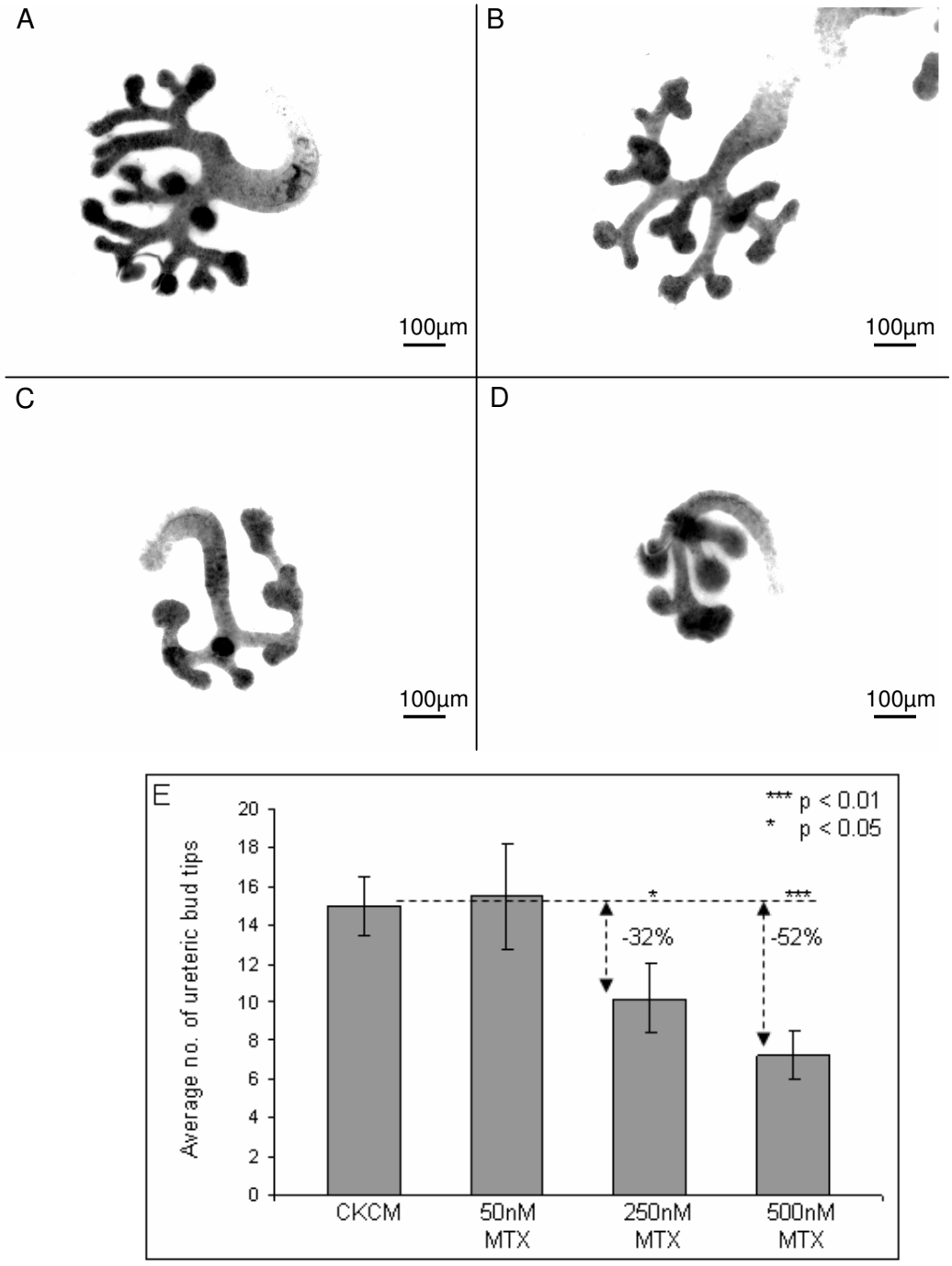


Fig.3.9 The effects of proliferation-inhibition on kidney branching.

Fig.3.9 shows E11.5 kidneys that were dissected and cultured in various concentrations of methotrexate (MTX) (SIGMA, M8407). After 96hrs of culture, the kidneys were fixed in methanol and stained for Calbindin-D28K. (A) Control kidneys cultured in CKCM, n=5 kidneys. (B) Kidneys cultured in CKCM with 50nM MTX, n=4 kidneys. (C) Kidneys cultured in CKCM with 250nM

MTX, n=5 kidneys. (D) Kidneys cultured in CKCM with 500nM MTX, n-5 kidneys. (E) Bar chart showing the mean values ± SEM of the number of ureteric bud tips for kidneys cultured in each culture condition. Data was analysed separately using unpaired one-tailed Student's t-test. T-test p-values for: CKCM vs. 50nM MTX p= 0.44, CKCM vs. 250nM MTX p= 0.04, CKCM vs. 500nm MTX p= 0.002. The colour in images (A-D) has been inverted for contrast and display purposes.

3.2.8.2 Inhibition of proliferation might account for Rac1 inhibitor effects on branching and growth.

I have shown that directly blocking proliferation with MTX reduces ureteric bud branching. This answers question (A) in the previous section. I also showed that a 250nM of MTX reduces branching similarly to 75µM NSC23766. This completes question (B). Question (C) asked whether it is possible to phenocopy the effects of Rac1 by just inhibiting proliferation. To answer this questions it is necessary to quantify the decrease in proliferation produced by 250nM MTX and 75µM NSC23766. If 75µM of the Rac1 inhibitor reduces both branching and proliferation equally to 250nM MTX, this would suggest that the reduced branching is solely an effect of decreased proliferation.

Experiments were set up where kidney cultures were maintained for 46hrs in control conditions, 75µM NSC23766, 150nM MTX or 250nM MTX. Kidneys were fixed and stained for H3(p), E-cadherin and nuclei marker TO-PRO-3. Proliferation were quantified using the H3(p) protocol where the number of mitotic cells was expressed as a percentage of the total number of nuclei present on each section analysed.

In control conditions (Fig.3.10A,E), 3.27% of detectable nuclei were positive for H3(p), and thus in mitosis. This value corresponds quite well with previously published mitotic indeces for the kidney (Foley and Bard 2002) where the authors used nuclear morphology, as visualised by Propidium Iodide stains, as an indicator of cells in mitosis. The mitotic index described therein was 2.1% (Foley and Bard 2002). Significantly, their work was carried out on kidneys not cultured in vitro but directly dissected for fixation and staining. Differences in the protocol and the mouse strains as well as the serum used in my experiments could account for the slightly higher proliferation rate recorded in my samples. The proliferation rate for kidneys in control conditions was determined by analysis of 18 sections from 9 kidneys. Quantification of proliferation rates in kidneys cultured in 75µM NSC24766 was previously done using BrdU. Here it was repeated using the H3(p) method. Kidneys cultured in 75µM NSC24766 contained an average of 2.16% of cells that were in

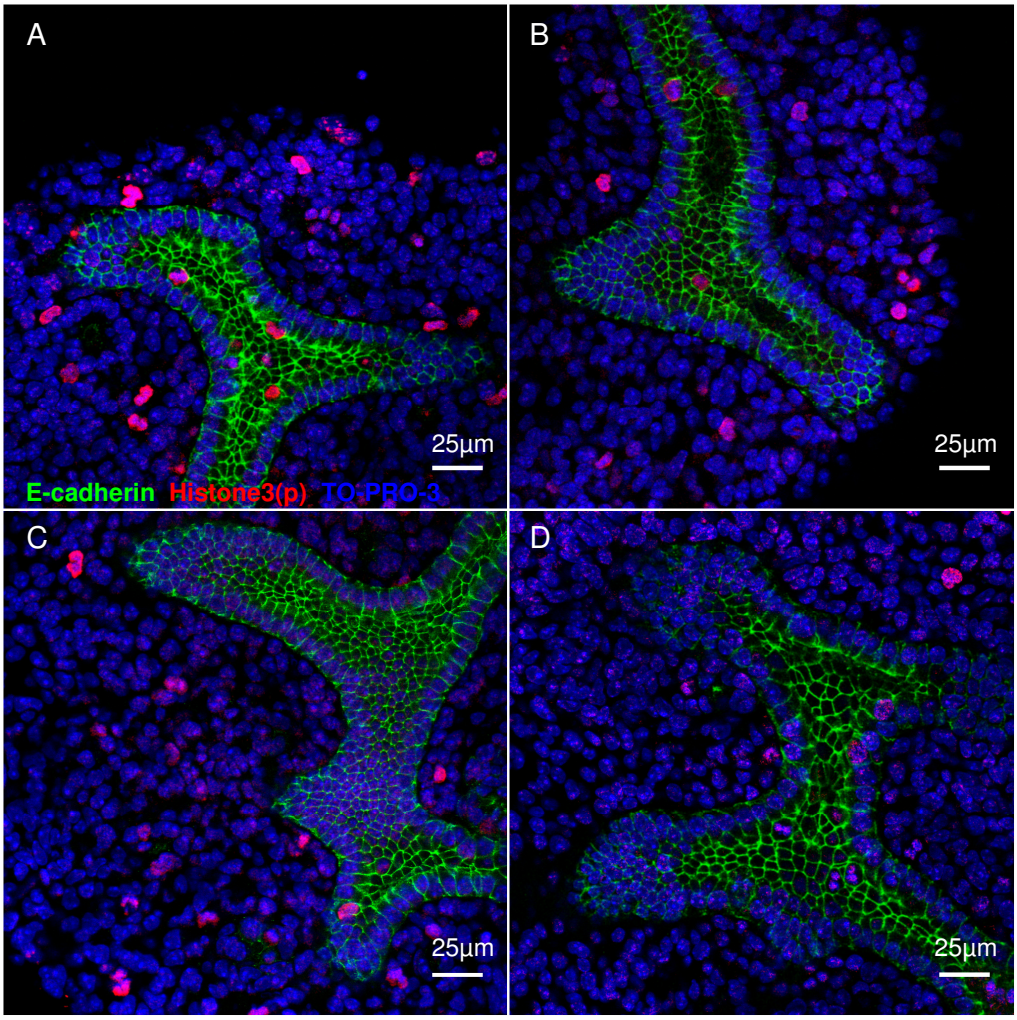
mitosis (Fig.3.10B,E). This represents a 34% reduction in the number of cells in mitosis compared to those kidneys in control conditions, p=0.0088. The same number of slides and kidneys were analysed for the samples cultured in 75µM NSC23766 as in controls. The P-values were calculated using unpaired one-tailed Student's t-tests with the standard error of mean (SEM) as the indicator of sample variability. This decrease in proliferation is similar to that described when using BrdU to detect proliferating cells. There, the decrease of BrdU incorporation was estimated to be 20-24%.

The analysis of kidneys cultured in 150nM (Fig.3.10C) or 250nM (Fig.3.10D) MTX showed that 2.73% and 1.34% of cells were in mitosis (Fig.10E). These percentages represents reductions of 18% (p=0.069) and 58% (p=0.00029) compared to controls, respectively. For the 150nM and the 250nM concentrations, 12 slides and 6, from 6 and 3 kidneys were analysed, respectively. The percentage of mitotic cells in control conditions, 75µM NSC23766, 150nM MTC and 250nM MTX were thus: 3.27%, 2.16%, 2.73% and 1.34%. Neither of mitotic indeces for the MTX cultures was equal to that of the 75µM NSC23766 cultures.

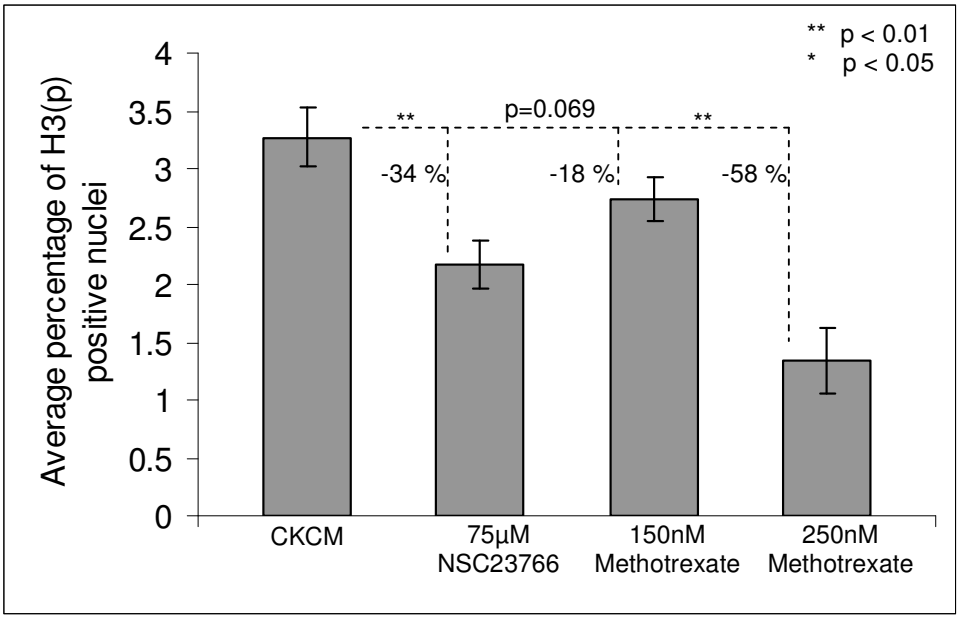
The number of ureteric bud tips was counted for each condition by use of the E-cadherin stain which stained the ureteric bud. Kidneys cultured in control conditions, 75µM NSC23766, 150nM MTX and 250nM MTX had an average of 21.9, 15.2, 12.43 and 6.4 ureteric bud tips (Fig.3.10F). Compared to control conditions, this represents reductions by 31%, 43% and 70% for the kidneys cultured in Rac1 inhibitor cultures and the two MTX cultures, respectively.

These results show that inhibiting Rac1 reduces branching less than what would be expected if Rac1 only controlled proliferation in the ureteric bud. It also shows that inhibition of proliferation does not produce a linear decrease in branching. It is difficult to explain why the Rac1 inhibitor inhibits proliferation more than 150nM MTX but decreases branching to a lesser extent. Firstly, Rac1 might be necessary as a negative regulator of branching through an unknown mechanism as well as being needed for proliferation. The inhibition of Rac1 suggests that proliferation is decreased and at the same the branching is somehow rescued. Secondly, Rac1 and

MTX might affect proliferation differently in the metanephric mesenchyme and in the ureteric bud. Diffusion problems might be the simples answer to this. MTX and Rac1 inhibitor NSC23766 have similar molecular weights of 454g and 531g, respectively, but their diffusion dynamics might be very different. Here I demonstrated that the Rac1 inhibitor reduces the proportion of cells progressing through DNA synthesis and mitosis in the ureteric bud tips and in whole sections of the kidney thus suggesting a decrease in actual proliferation rates. This suggests that the Rac1 inhibitor reaches the ureteric bud. However these results could be an indirect of a process affected in the mesenchyme. For example, a reduction in metanephric mesenchyme proliferation could lead to a reduction in available space for the ureteric bud to branch. The MTX might on the other hand affect both the metanephric mesenchyme and ureteric bud proliferation directly. It is not know how proliferation is regulated in the ureteric bud tips although inhibition of the Erk/MAPK pathway and GDNF reduces the ureteric bud tip proliferation (Fisher et al 2001; Michael and Davies 2004).


A
B
C
D
25µm
E-cadherin Histone3(p) TO-PRO-3

E


** p < 0.01
* p < 0.05
Average percentage of H3(p) positive nuclei
0
0.5
1
1.5
2
2.5
3
3.5
4
**
p=0.069
**
-34 %
-18 %
-58 %
CKCM
75µM NSC23766
150nM Methotrexate
250nM Methotrexate

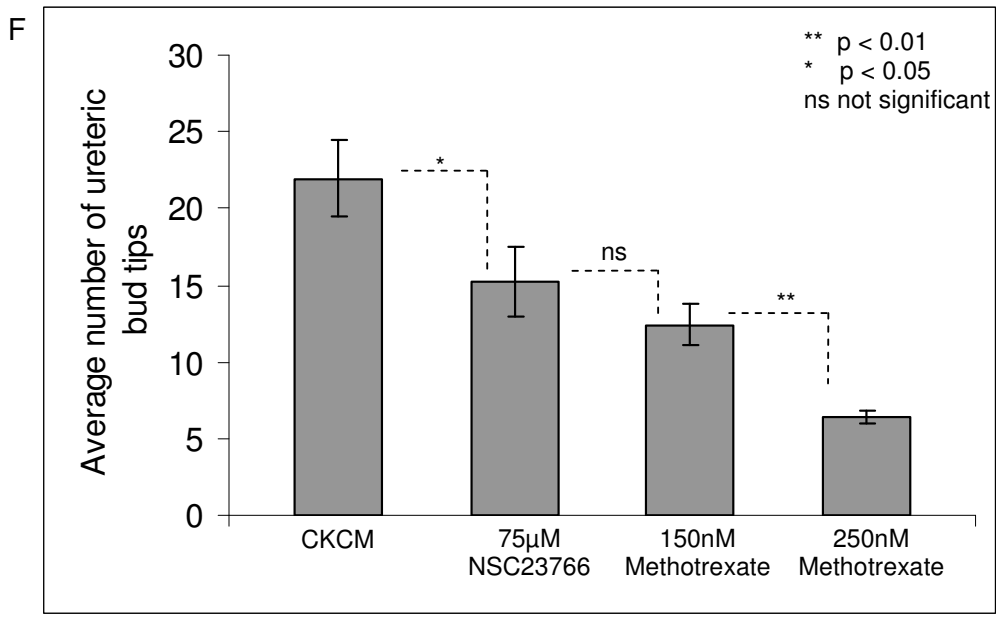


Fig.3.10 The effects of Rac1 inhibition on kidney proliferation analysed using H3(p) and Methotrexate.

Fig.3.10 shows kidneys that were cultured in (A) CKCM, (B) with 75µM NSC23766, (C) with 150nM Methotrexate or (D) with 150nM Methotrexate for 48hrs, fixed in methanol and stained for mouse-E-cadherin and rabbit-phosphorylated histone 3. These were detected with anti-mouse-FITC and anti-rabbit-TRITC. Kidneys were stained with TO-PRO-3. (E) Bar chart showing the mean percentage of nuclei positive for phosphorylated histone 3 out of the total number of nuclei ± SEM. Data was analysed separately using unpaired one-tailed Student's t-tests. T-test p-values for: CKCM vs. 75µM NSC23766 p= 0.0088 (18 slides per group, 9 kidneys per group), CKCM vs. 150nM Methotrexate p = 0.069 (18 and 12 slides, 9 and 6 kidneys per group, respectively) CKCM vs. 250nM Methorexate p =0.00029 (18 and 6 slides, 9 and 3 kidneys per group, respectively). (F) Bar chart showing the mean number of ureteric bud tips ± SEM. Data was analysed separately using unpaired one-tailed Student's t-tests. T-test p-values for: CKCM vs. 75µM NSC23766 p= 0.031 (n= 11 and 10 kidneys, respectively), 75µM NSC23766 vs. 150nM Methotrexate p = 0.18 (n=10 and 7 kidneys, respectively). 75µM NSC23766 vs. 250nM Methotrexate p = 0.0022 (n= 10 and 5 kidneys, respectively).

3.2.8.3 Inhibition of Rac1 or proliferation does not affect ureteric bud cell size.

The reduced branching and reduced kidney size in cultures where Rac1 is inhibited have been shown to be regulated by affects on proliferation. There is another possibility that could result in a decrease in the overall size of the kidneys. A decrease in the general cell size could result in smaller kidneys. As mentioned before, it has previously been shown that a planar cell polarity regulates the size of nephron progenitor cells in two dimensional in vitro cultures (Osafune et al 2006). It is thus necessary to determine whether a decrease in cell size is accountable for the decrease in branching or kidney size in cultures treated with Rac1 inhibitor.

Using the data that has previously been described in (Fig.3.10), it was possible to measure the cell width of cells in the ureteric bud. For a more detailed description of measurements of the cell width, see Chapter 4 where the cell width in the ureteric bud is discussed in relation to Rho-kinase. The cell width was measure in the ureteric bud from kidneys cultured in control conditions, 75µM NSC23766 or 150nM MTX. Table 3.3 shows the average width of the ureteric bud cells in each condition.

Condition	**Cell count**	**Average width (µm ±StDev)**
Controls	89	6.55 ± 1.7
75µM NSC23766	90	6.5 ± 1.6
150nM MTX	110	6.4 ± 1.7

Table 3.3 Ureteric bud cell width

Ureteric bud cell width is not affected by 75µM NSC23766 compared to controls ($p=0.44$), nor is it affected by a decrease in proliferation by 150nM MTX ($p=0.26$). In fact, the cell width is remarkably consistent both within every sample as well as between kidneys in different conditions. Cell width can however be affected by specific proteins as will be discussed in Chapter 4. These data supports the suggestion that decreased proliferation is at least one of the major causes for the decrease in branching and growth of kidneys where Rac1 is inhibited.

3.3 Chapter discussion

3.3.1 Evidence of Rac1 affecting nephrogenesis

The only data that support a role for Rac1 during nephrogenesis come from the demonstration that JNK1, JNK2 and Rac1 regulates the size of cultures of nephron progenitor cells (Osafune et al 2006). This data from Osafune and colleagues is based a novel method where a population of Sall1-positive cells from the metanephric mesenchyme are cultured on a layer of layer of 3T3 cells expressing Wnt4 (Osafune et al 2006). The cultures were derived from single cells from the metanephric mesenchyme that in culture demonstrated the potential to express a large array of genes expressed in the nephron suggesting that they are multipotent and can differentiate down a wide array of nephron lineages. Inhibition of either JNK1 or JNK2 disrupted the 'maturation'/differentiation of these cultures and the inhibition of JNK1, JNK2 or Rac1 results in decreased culture sizes (Osafune et al 2006). Inhibition of JNK1 or JNK2 also resulted in smaller kidneys in culture. The authors suggested that Rac1, JNK1 and JNK2 all participate in a planar cell polarity pathway which acts to regulate nephron differentiation and kidney and nephron size.

I investigated whether the inhibition of Rac1 affects nephron development in vitro using whole kidney cultures and cultures of spinal cord induced metanephric mesenchyme. No evidence that was gathered suggests that Rac1 affects the differentiation of nephrons in culture. Equally, the size of nephrons in culture appears to be unaffected by the inhibition of Rac1.

It is unclear whether these results are in contradiction. Osafune and colleagues never showed whether Rac1 affects the differentiation of nephron progenitor cells, and it is very difficult to determine whether the size of nephrons are affected in whole kidney cultures as previously explained. It would be of interest to determine whether the results obtained by Osafune and colleagues are replicable in whole kidney cultures or in transgenic models for in vivo studies. Equally, it would be interesting to determine whether a planar cell polarity pathway is specifically used in nephron progenitor cell populations in the kidney. Since it is possible to alter genes conditionally in a particular subset of cells, one attractive line of investigation would be to delete JNK1

or Rac1 conditionally from the nephron progenitor cell population, as done for β-catenin by Park, Valerius and McMahon (Park, Valerius, and McMahon 2007).

3.3.2 Rac1 regulating proliferation and ureteric bud branching in the kidney

Proliferation in the kidney has previously been shown to be regulated, at least in part, by GDNF/c-Ret signalling (Michael and Davies 2004). The Rac1 and RhoA pathways have both been implicated in regulating the size of kidneys in culture (Meyer et al 2006; Osafune et al 2006). RhoA and Rac1 can both be activated by the Wnt-Frizzled planar cell polarity pathway (Habas, Dawid, and He 2003). Interestingly, in the kidney Rac1 and RhoA appear to promote opposite regulations of kidney growth. Whereas inhibition of RhoA or Rho-kinase results in increased culture sizes (Meyer et al 2006; Osafune et al 2006), the inhibition of Rac1 has the opposite effect (Osafune et al 2006). Inhibition of Rho-kinase has been demonstrated to result in increased proliferation in whole kidney cultures (Meyer et al 2006). The inhibition of Rac1 has not previously been carried out in whole kidney cultures, nor has the effects of Rac1 been characterized.

I show that Rac1 is necessary to maintain normal proliferation in the kidney. Inhibition of Rac1 also results in reduced branching of the ureteric bud although it is uncertain whether this is a direct effect from the reduced proliferation. It is however possible to reduce branching by directly limiting proliferation. Proliferation has previously been linked to ureteric bud induction and also as a possibly mechanism for branching (Michael and Davies 2004). This data supports the hypothesis that proliferation is a plausible mechanism for ureteric bud branching although it does in no way prove it. An interesting follow-up to these experiments would be to determine whether the direct inhibition of proliferation, exclusively in the ureteric bud tips would result in reduced terminal bifurcations. Specific inhibition of proliferation in the ureteric bud tips, using for example a *Wnt11-Cre* and conditional *Rac1* would provide great insights not only to whether Rac1 regulates proliferation in the ureteric bud tips, but also whether branching would now occur at all.

3.3.3 Rac1 and cellular migration in the kidney

The prospect of Rac1 regulating a mechanism of cellular motility stemmed from research indicating such a process in the ureteric bud (Watanabe and Costantini 2004; Michael, Sweeney, and Davies 2005; Meyer et al 2006; Kim and Dressler 2007). Unlike the pten mutant described by Kim and Dressler (Kim and Dressler 2007), I found no evidence that the inhibition of Rac1 disturbed the basement membrane at the ureteric bud tips. From experience, the basement membrane at the tips does however display quite a variable appearance as would be expected from such as dynamic structure. The evidence of cell scattering from the ureteric bud tips of kidneys treated with cytochalasin D (Michael, Sweeney, and Davies 2005) is likely to be an artefact of cell death. In a preliminary study, I attempted to replicate these results and found that the cell scattering effect is difficult to replicate in a reliable manner. Those 'cells' that did scattered were briefly analysed using propidium bromide as done by Foley and Bard (Foley and Bard 2002) and the cells displayed nuclei that were clearly apoptotic (data not included). It is however possible that apoptosis was a consequence rather than the effect that resulted in cell scattering. Migration is still an intriguing possibility in particular because many of the molecular cues needed for cellular motility are present in the kidney. Rac1 has been demonstrated to be important for tubulogenesis from MDCK cell cysts (Rogers et al 2003). However, since this process is dependent on polarity rearrangements and on the actions of single cells it is not clear whether this can be extended to suggest that Rac1 might be important for branching in the ureteric bud as neither of the processes described, is evident. Further studies would be required to determine whether cellular motility is indeed a mechanism of branching.

Chapter 4

Rho-kinase, a regulator of nephrogenesis, nephron maturation and proximal-distal patterning

4.1 Introduction

4.1.1 Overview

RhoA-GTPase is a sister protein to Rac1-GTPase which is discussed in Chapter 3. Rac1-GTPase (Rac1) and RhoA-GTPase (RhoA) are important for several common processes such as regulation of migration, adhesion and polarity. This chapter focuses on the RhoA-pathway and in particular on the RhoA effector Rho-kinase. The importance of Rho-kinase (ROCK) for the development of the metanephric nephron has not previously been reported, although ROCK has been investigated in the metanephric kidney (Michael, Sweeney, and Davies 2005; Meyer et al 2006) and in renal progenitor cell cultures (Osafune et al 2006). These investigations have used soluble cell permeable ROCK inhibitors that have been administered directly via the medium to kidneys or kidney tissues in culture. Data have suggested that inhibition of ROCK in renal progenitor cell cultures has no effect on cell differentiation but possibly acts via a planar cell polarity pathway to regulate culture size (Osafune et al 2006). These effects on culture size have also been shown in whole kidney cultures where the inhibition of ROCK results in higher rates of proliferation (Meyer et al 2006). The aim of this chapter is to discuss the possibility that ROCK regulates several other aspects of kidney development. In the introduction I will focus on the role of ROCK in stress-fibre formation and the regulation of cell adhesion and cell polarity as these are likely to be important factors regulating ureteric bud morphogenesis and in particular nephrogenesis and nephron maturation.

4.1.2 The Rho-ROCK pathway: stress-fibres

The RhoA-GTPase pathway branches out to exert control over a large number of processes including regulation of proliferation, adhesion, and stress-fibre formation. Downstream effectors such as Citron, protein kinase (PKN), Rho-kinase (ROCK) and Diaphanous homologues (mDia) are involved in the regulation of these processes, reviewed by Schwartz (Schwartz 2004). In this chapter the focus is on the

signalling pathway leading from RhoA to its kinase ROCK, and the closely connected Dia1 pathway which together regulate stress fibre formation (Watanabe et al 1999); the pathway is outlined in (Diagram4.1). The ROCK pathway diverges to regulate the activity of proteins such as Lin11, Isl1 and Mec3 Kinase (LIMK), myosin light chain (MLC) and myosin light chain phosphatase (MLCP) (Amano et al 1996; Kimura et al 1996; Kawano et al 1999; Maekawa et al 1999) as well as ezrin, radixin and moesin (ERM) proteins, sodium-hydrogen exchanger 1 (NHE1) and Adducin, as reviewed by Riento and Ridley (Riento and Ridley 2003). The proteins regulated by RhoA and ROCK for the formation and contraction of actin-myosin are LIMK, MLC, MLCP and mDia1. RhoA activates ROCK (Matsui et al 1996) and ROCK activates MLC by phosphorylation primarily on Ser19 (Amano et al 1996). The activation of MLC results in its interaction with actin and the activation of myosin ATPase, resulting in the contraction of non-muscle actin-myosin (Amano et al 1996). Rho-kinase also phosphorylates MLCP, an inhibitor of MLC, and thus inhibits its activity and increases the levels of phosphorylated MLC (Kimura et al 1996; Kawano et al 1999). MLC is also activated by myosin light chain kinase (MLCK), through the phosphorylation of MLC on Thr18 and Ser19, discussed by Amano and colleagues (Amano et al 1996). MLC is negatively regulated by Protein kinase C (PKC) which phosphorylates MLC on Ser1 Ser2 and Thr9, also discussed by Amano and colleagues (Amano et al 1996).

The connection between ROCK and the stability of the actin cytoskeleton has been demonstrated to occur via LIMK and cofilin (Maekawa et al 1999). ROCK phosphorylates LIMK which in turn phosphorylates cofilin and stops its actin depolymerising activity (Maekawa et al 1999). This presumably results in the inhibition of actin disassembly and the stabilisation of the actin-myosin stress-fibres. mDia1 is also activated by RhoA, and it acts in parallel with the ROCK pathway (Watanabe et al 1999). In fact, it seems as if ROCK and mDia1 act together, where ROCK regulates myosin phosphorylation, and thus the capacity to contract, whereas Dia1 regulates, through profilin, the actin polymerisation (Watanabe et al 1999). It has been demonstrated that Dia1 produces thin actin fibres and depending on the ratio of Dia1 and ROCK, the thickness and morphology of the stress-fibres can be

regulated which might have an impact on the function of the stress-fibres (Watanabe et al 1999). As mentioned, ROCK also regulates other proteins such as the ERM proteins, NHE1 and Adducin, which has recently been reviewed by Riento and Ridley (Riento and Ridley 2003).

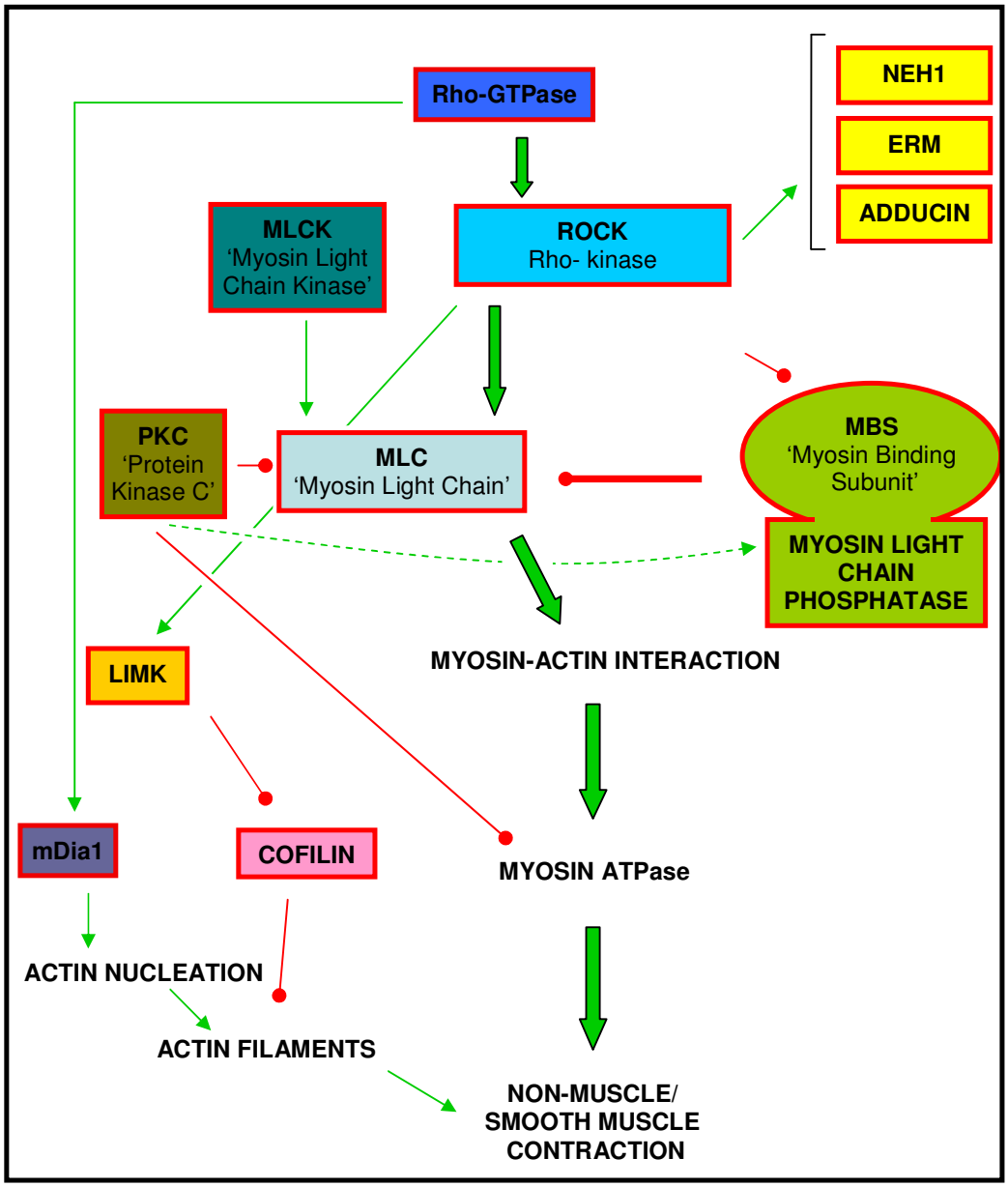


Diagram4.1 The Rho-kinase pathway (protein interactions are as described and cited in the text).

4.1.2.1 ROCK: adhesion and polarity

RhoA induces the formation of focal adhesion complexes and regulates adherens junctions (Ridley and Hall 1992; Sahai and Marshall 2002). The stress fibres formed by ROCK are in fact linked to the extracellular matrix via integrins in focal adhesion complexes (Ridley and Hall 1992; Nobes and Hall 1995). Activation of RhoA results in the formation of focal adhesions (Nobes and Hall 1995) a process acting via ROCK (Amano et al 1997). A particularly interesting property of the Rho-ROCK/Dia1 pathway is its ability to regulate adherens junctions. It has been demonstrated that the physical force exerted by actin-myosin contractions can result in the disruption of adherens junctions and that the activity of Dia1 can stabilise them (Sahai and Marshall 2002); see Diagram4.2. Adherens junctions consist of several components including β-catenin which is bound to cadherins, as reviewed by Fukata and colleagues (Fukata et al 1999). This has an additional level of interest since the canonical β-catenin signalling pathway has been demonstrated during nephron development and ureteric bud tip identity maintenance (Park, Valerius, and McMahon 2007; Marose et al 2008).

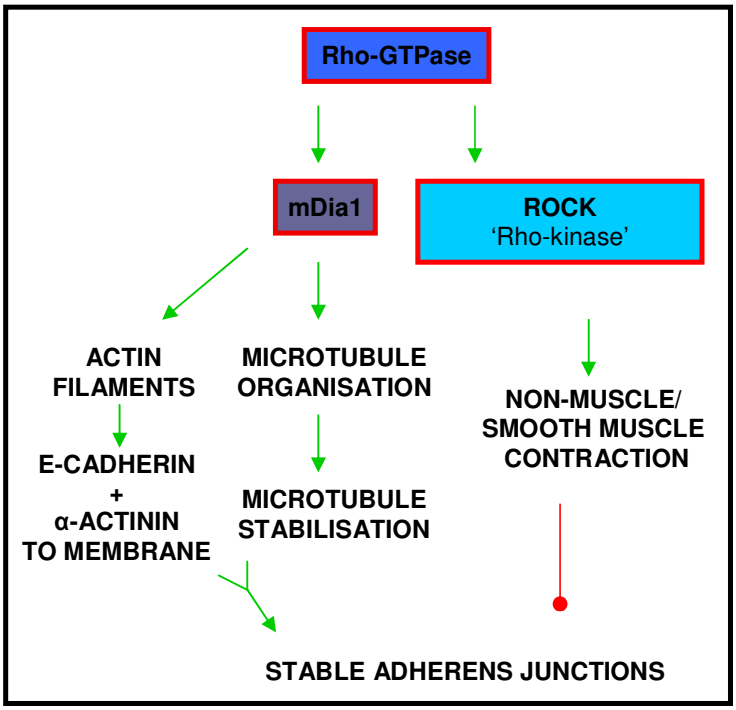


Diagram4.2 ROCK and Adherens Junctions (based on Fig.7 in Sahai and Marshall 2002).

The Rho-ROCK pathway has been studied mainly in vitro using cell culture systems. A system relevant to the kidney is the culture technique where Madin-Darby Canine Kidney (MDCK) cells are seeded into an extracellular matrix gel and undergo both cystogenesis and tubulogenesis (Montesano, Schaller, and Orci 1991). Several studies have determined that RhoA can regulate apicobasal polarity (Rogers et al 2003; Yu et al 2008). MDCK cells expressing a dominant-negative *RhoA* have been shown to display inverted apicobasal polarity (Rogers et al 2003), although in other studies, neither the expression of *ROCK-I*, *ROCK-II* or *RhoA* shRNA nor inhibition of ROCK with moderate levels of ROCK inhibitor disturbed normal polarity (Yu et al 2008). Epithelial cells that are developing an apicobasal polarity require polarity cues from the extracellular matrix. MDCK cells in gel cultures have been shown to use β1-integrin, which in turn can activate Rac1, discussed by Yu and colleagues (Yu et al 2008). Blocking β1-integrin resulted in reduced Rac1 activity, increased RhoA activity and inverted apicobasal polarity, the latter which could be rescued by the expression of *ROCK-I* or *RhoA* shRNA as well as by inhibition of ROCK or myosin II (Yu et al 2008). Similarly, the expression of dominant negative *Rac1* in MDCK cells resulted in inverted apicobasal polarity (Rogers et al 2003; Yu et al 2008), which could also be rescued by the inhibition of the RhoA-pathway (Yu et al 2008).

An important point is that the effects on polarity that were produced through the inhibition of the RhoA-pathway or β1-integrin were only potent during the first 3 days of culture. Thereafter, inhibition of β1-integrin could not invert polarity, and equally the 'rescue' of normal polarity in β1-integrin blocked cysts, by inhibiting the RhoA-pathway, could not occur (Yu et al 2008). This suggested that, rather than continuously regulating the polarity, these proteins are important for the establishment of polarity (Yu et al 2008); a point that will be important at a later point in this chapter. However, the inverted polarity produced by the expression of dominant negative *Rac1* in MDCK cell cysts was reversible even after 4 days of inverted polarity if the dominant negative *Rac1* expression was stopped (O'Brien et al 2001). The inversion produced through dominant negative *Rac1* lead to abnormal laminin deposition on the basal surface of MDCK cell cysts (O'Brien et al 2001). Although the apicobasal inversion in MDCK cell cysts expressing dominant negative

Rac1 resulted in apical makers being detected on the basal surface, basal laminin was not deposited inside the cysts (O'Brien et al 2001).

The RhoA-pathway has been shown to be important in whole organs as well as in cell cultures. In mammary glands, in culture large-scale epithelial reorganisations are required (Ewald et al 2008). Mammary glands in gel cultures initiate duct formation in a Rac1 and proliferation dependant manner where the growing ducts display a non-polarised, multi-layered epithelial structure (Ewald et al 2008). During maturation and establishment of proper ducts the epithelium becomes polarised and expresses markers for apical and basal surfaces (Ewald et al 2008). The organisation of this polarity has been demonstrated to be dependent on ROCK, as the inhibition of ROCK results in persistently multi-layered, and overly branched ducts with abnormal apicobasal polarity (Ewald et al 2008). No mechanism has been proposed to explain these effects apart from abnormal cell morphology (Ewald et al 2008).

The RhoA-pathway has also been shown to be important for polarity in terms of regulating the stability of the basement membrane (Nakaya et al 2008). During gastrulation, presumptive mesodermal cells in the epiblast down-regulate RhoA activity in order to break down the adjacent basal membrane so that they can detach and ingress (Nakaya et al 2008). The basement membrane was shown to be controlled by RhoA positively regulating microtubule stability (Nakaya et al 2008).

These examples demonstrate that RhoA is in fact involved in a wide array of process that regulate polarity and in some cases this has been shown to be controlled via ROCK, which can maintain or establishment a proper apicobasal polarity.

4.1.2.2 ROCK I, ROCK II: knockouts and drugs

Two very closely related ROCK proteins exist: ROCK-I (formerly known as ROCKβ and p160ROCK) and ROCK-II (formerly known as ROKα and Rho-kinase), reviewed by Riento and Ridley (Riento and Ridley 2003). The ROCK inhibitors used

in this thesis inhibit both of the ROCK proteins. The ROCKs consist of a kinase domain (with 92% similarity), a Rho-binding domain (highly similar) and pleckstrin-homology domains (65-70% similarity), reviewed by Riento and Ridley, and Yoneda, Multhaupt and Couchman (Riento and Ridley 2003; Yoneda, Multhaupt, and Couchman 2005). ROCK-I and ROCK-II regulate different myosin dependent processes. ROCK-I is necessary for MLC and MLCP phosphorylation leading to stress-fibre and focal adhesion formation, processes in which ROCK-II is not normally involved (Yoneda, Multhaupt, and Couchman 2005). ROCK-II on the other hand regulates phagocytosis and also has an effect on both stress fibres and focal adhesions although these effects are not fully characterised (Yoneda, Multhaupt, and Couchman 2005). ROCK-I and ROCK-II also play different roles during fibronectin matrix assembly, but this might be linked to the differences in their roles in regulating stress-fibres (Yoneda et al 2007).

Knockouts of been made of both *ROCK-I* (Shimizu et al 2005) and *ROCK-II* (Thumkeo et al 2003). The *ROCK-I* knockout died after birth and displayed open eyelids at birth together with omphalocele (Shimizu et al 2005). The defects seen in eyelid closure were speculated to be a result of abnormal actin-myosin cable formation in the eyelids during development (Shimizu et al 2005). No obvious abnormalities were detected in the majority of embryonic organs, including the kidney (Shimizu et al 2005). The *ROCK-II* knockout had placental defects and died after E13.5. As well as placental defects, the other obvious abnormality was haemorrhaging in the capillaries of the hind limbs (Thumkeo et al 2003). Interestingly, no phenotypes were detected in the majority of organs, including the kidney. In cell cultures from the *ROCK-II*$^{-/-}$ mice, the authors demonstrated that the *ROCK-II*$^{-/-}$ mutation was not sufficient to cause alterations of the actin cytoskeleton; however, upon the addition of ROCK-inhibitor, the cytoskeleton became highly abnormal (Thumkeo et al 2003). It was suggested that this was a result of ROCK-I being inhibited by the ROCK-inhibitor as well as *ROCK-II* being absent (Thumkeo et al 2003).

Although there is some evidence that ROCK-I and ROCK-II play non-redundant roles, it is clear that the interplay between ROCK-I and ROCK-II is not well understood as the inhibition of ROCK-II affects the ROCK-I regulated actin-myosin cables and focal adhesions by an unidentified mechanism (Yoneda, Multhaupt, and Couchman 2005). To my knowledge no compound knockouts of ROCK-I and ROCK-II have yet been made, but in cells doubly targeted with ROCK-I and *ROCK-II* siRNA, these resemble ROCK inhibitor treated cells (Yoneda, Multhaupt, and Couchman 2005). It is likely that double mutants, ROCK inhibitors and specific targeting using conditional mutants, will yield much more informative data.

As mentioned, soluble cell-permeable inhibitors of ROCK-I and ROCK-II have been used in this thesis; these inhibitors are Y-27632 and glycyl-H1152 dihydrochloride (H1152). These have previously been used for organ culture studies (Michael, Sweeney, and Davies 2005; Meyer et al 2006; Ewald et al 2008). Uehata and colleagues identified the molecular compound Y-27632 as an effective inhibitor of smooth muscle contraction, focal adhesion formation and stress fibre formation in HeLa cells and in tissues such as smooth muscle in the vasculature and in the respiratory system (Uehata et al 1997). Y-27632 was shown to bind ROCK-I and ROCK-II with an affinity in excess of 200-2000 times that for binding to other kinases (Uehata et al 1997). In addition, Y-27632 did not inhibit PAK, which is important for Rac1 and Cdc42 signalling, as previously discussed by Westwick, and by Rosenfeldt (Westwick et al 1997; Rosenfeldt et al 2006). Y-27632 inhibits ROCK-I and ROCK-II by competing with ATP for binding (Ishizaki et al 2000). ROCK-I and ROCK-II can thus not be activated upon binding to Y-27632 (Ishizaki et al 2000). It is also speculated that the binding of Y-27632 to ROCK-I and ROCK-II results in conformational changes of the proteins, although this has not been formally demonstrated (Ishizaki et al 2000). The second ROCK-I and ROCK-II inhibitor glycyl-H1152 dihydrochloride: (S)-(+)-4-Glycyl-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1H-1,4-diazepine dihydrochloride (H1152) also functions by competing for ATP and thus ROCK-I and ROCK-II activation (Sasaki, Suzuki, and Hidaka 2002). It is identified as compound 18 described by Tamura and colleagues (Tamura et al 2005). H1152 is approximately 15 times more potent in

inhibiting ROCK-I and ROCK-II compared to Y-27632, reviewed by Tamura and colleagues (Tamura et al 2005). Table 4.1 displays Inhibitor Concentration 50% (IC_{50}) values (in µM) for 4 common ROCK inhibitors derived from in vitro assays. The table and the data were adapted from Tamura et al 2005. IC_{50} values for H1152 were assumed to be those of compound 18 described in (Tamura et al 2005), based on the identical but only partial values listed for H1152 by the distributor (Catalogue number 2485/TOCRIS).

It is important to point out that the concentrations stated in Table 4.1 reflects the IC_{50} values from purified protein. These values can therefore not be directly used directly to estimate the concentrations required for in vitro, cell or organ cultures, which will need significantly higher values. The IC_{50} values in Table 4.1 should be used comparatively rather than be used as absolutes or indications. I have used Y-27632 concentrations ranging from 5µM-20µM as used by Michael, Sweeney and Davies (Michael, Sweeney, and Davies 2005). H1152 concentrations were used for the same effects at concentrations ranging from 0.625-2.5µM, i.e. ~15x less as is specified in Tamura et al 2005.

ROCK-I and ROCK-II are both found in the kidney in the ureteric bud and in the metanephric mesenchyme (Meyer et al 2006). The distribution of ROCK-II was also confirmed in my results (Appendix 2-Fig.A2.1). *RhoA-GTPase*, *ROCK-I* and *ROCK-II* have also been annotated as being present in the majority of structures in the metanephric kidney (www.GUDMAP.org).

The work described in Chapter 4 aims to show that ROCK is a necessary component of the pathway that drives epitheliogenesis and which gives rise to the nephrons, as well as for the mechanism that regulates the morphological maturation of the nephron. The following work is currently in preparation for publication.

Inhibitor (IC_{50} µM)	Y-27632	H1152 dihydrochloride	Glycyl-H1152 dihydrochloride	HA-1077
ROCKII	0.162	0.012	0.0118	0.158
PKA	>100	3.03	>10	4.58
PKC	25.8	5.68	>10	12.3
PKG	3.27	0.36	3.26	1.65
MLCK	>100	28.3		21.6
CaMKII	13	0.18	2.57	6.7
AuroraA	>100	0.745	2.35	100
GSK3alpha	50	60.7		>100
AMPK	>100	100		100
IKKalpha	>100	>100		100
CK2	>100	>100		>100
Erk1	>100	>100		>100
P38alpha	>100	100		>100
JNK1alpha1	>100	>100		>100
MKK4	>100	16.9		100
Abl	>100	7.77		>100
Src	>100	3.06		>100
EGFR	100	50		>100

KEY
<10x ROCKII IC_{50}
<100x ROCKII IC_{50}
<500x ROCKII IC_{50}
>5000x ROCKII IC_{50}

	[10x] ROCKII IC_{50}	[100x] ROCKII IC_{50}	[500x] ROCKII IC_{50}
Y-27632	1.620	16.200	81.000
H1152 dihydrochloride	0.120	1.200	6.000
Glycyl-H1152 dihydrochloride	0.118	1.180	5.900
HA-1077	1.580	15.800	79.000

Table 4.1: IC_{50} µM of common ROCK inhibitors.

The table and data were adapted from Tamura et al 2005. Glycyl-H1152 dihydrochloride has an IC_{50} µM concentration ~15 times lower compared to ROCK inhibitor Y-27632. The IC_{50} µM concentration of Glycyl-H1152 dihydrochloride towards ROCKII is ~200-280 times lower than that for other kinases Aurora A, CaMKII and PKG. The IC_{50} µM concentration of Y-27632 towards ROCKII is only >100, ~80 and ~20 times lower than that for kinases Aurora A, CaMKII and PKG, respectively.

4.2 Results

4.2.1 Rho-kinase regulation in normal kidney development.

To explore the possibility that Rho-kinase (ROCK) regulates aspects of de novo epithelial nephron formation, I employed in vitro kidney organ culture systems that are ideal for efficient targeting of specific proteins during tissue and organ development. These organ culture systems are the same as previously used for investigating the role of ROCK in the ureteric bud (Michael, Sweeney, and Davies 2005). ROCK inhibitor Y27632 has previously been used successfully for specific inhibition of ROCK function in organ culture (Michael, Sweeney, and Davies 2005; Meyer et al 2006; Ewald et al 2008) and a previous form of H1152, (H1152 dihydrochloride) which is not glycylated, has also been used (Michael, Sweeney, and Davies 2005). Reports have shown that ROCK is an essential regulator of kidney development, proliferation and in particular ureteric bud branching morphogenesis (Michael, Sweeney, and Davies 2005; Meyer et al 2006). The studies did detect nephron defects possibly as a result of the differences in techniques used; this will be elaborated on in section 4.2.3.

4.2.2 Rho-kinase inhibition on ureteric bud morphogenesis.

Using already optimised ROCK inhibitor concentrations for Y-27632 on whole kidney cultures, the initial experiments were aimed at replicating the previously observed ureteric bud morphology defects (Michael, Sweeney, and Davies 2005; Meyer et al 2006). Kidneys were cultured for 72hrs prior to fixation and staining with mouse anti-Calbindin D-28K. Kidneys were cultured either in control conditions/complete kidney culture medium (CKCM) or in CKCM with different concentrations of ROCK inhibitor: 0µM, 5µM, 10µM or 20µM Y-27632, (Fig.4.1A-D) respectively. The effects of Y-27632 on the ureteric bud morphology closely resemble those described in earlier reports (Michael, Sweeney, and Davies 2005; Meyer et al 2006). Specifically, the

ureteric bud increased in both width and overall area at the exposure to ROCK inhibitor Y-27632, an effect attributed to increased rates of proliferation (Meyer et al 2006). The form of H1152 used here, has been suggested to provide increased specificity and higher potency compared to Y-27632 (Tamura et al 2005), see Table 4.1 for additional details. From the IC_{50} data presented in Table 4.1, it was predicted that the concentrations of H1152 required to mimic those effects by Y-27632 would be around 15 times lower. To test whether H1152 would produce similarly disturbed ureteric buds at the predicted conditions, I set up identical experiments using H1152. H1152 was used at concentrations: 0µM, 0.625µM, 1.25µM or 2.5µM H1152, (Fig.4.2A-D) respectively. As can be seen from the morphology of the ureteric bud, a concentration of 20µM Y-27632 (Fig.4.1D) is similar to a concentration of 1.25µM H1152 (Fig.4.2C); this is 16 times lower. This suggests that the IC_{50} values presented in Table 4.1 were useful as a tool to indicate the concentrations of H1152 that would produce comparative results to the previously optimised Y-27632 concentrations.

These data show that both Y-27632 and H1152 produce the same morphological defects in the ureteric bud. Michael, Sweeney and Davies mentioned that Y-27632 and H1152-dihydrochloride produced different effects on proliferation where only H1152-dihydrochloride increased proliferation (Michael, Sweeney, and Davies 2005). Meyer and colleagues, on the other hand, published that Y-27632 also produced an increase in the proliferation rates in the kidney (Meyer et al 2006). My results suggest that Y-27632 and H1152 increases ureteric bud size equally at moderate concentrations, compare (Fig.4.1C) and (Fig. 4.2C). In this thesis both Y-27632 and H1152 have been used for the majority of experiments, and no differences were ever detected between the drugs apart from the ~15 fold difference in concentrations required to produce the same effects.

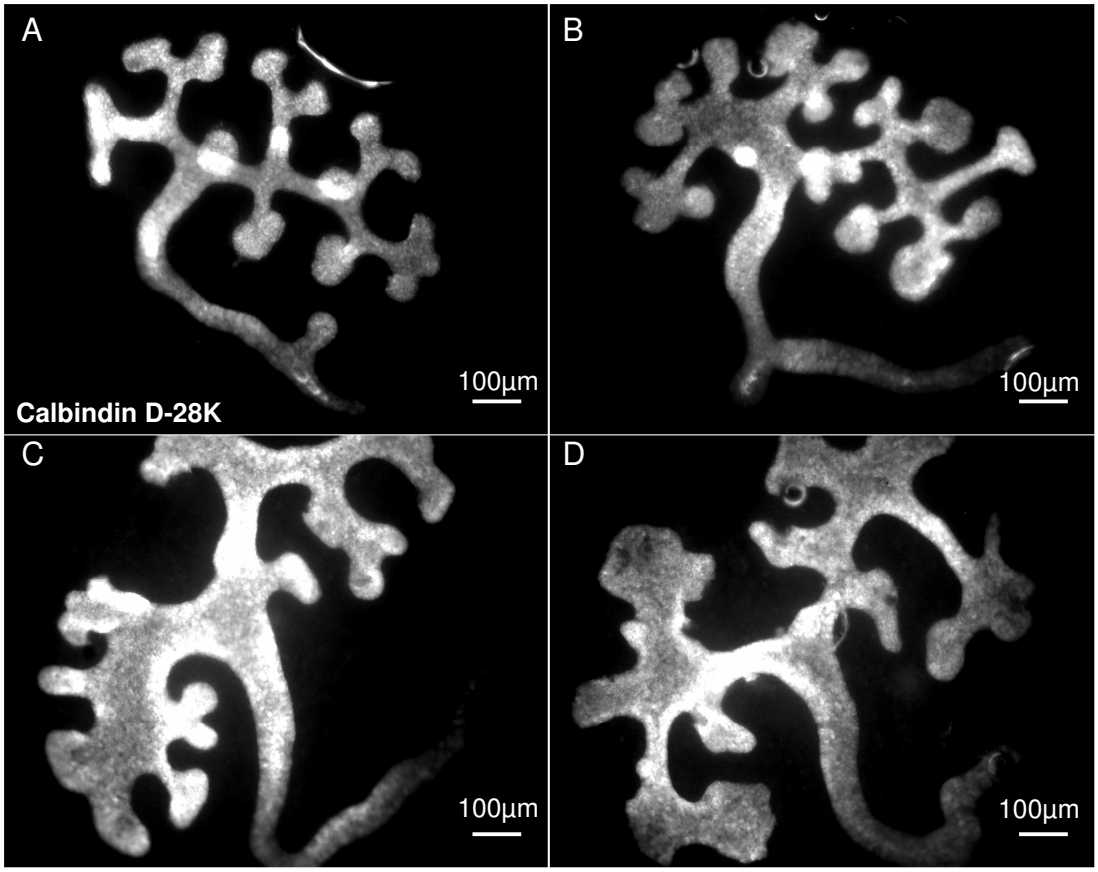


Fig.4.1 Inhibition of ROCK results in altered branching morphology.

Fig.4.1 shows E11.5 kidneys that were dissected and cultured for 48hrs. Varying concentrations of Y27632 Rho-Kinase Inhibitor were used. (A) Kidney cultured in CKCM. (B) Kidney cultured in CKCM supplemented with 5µM Y27632. (C) Kidney culture in CKCM supplemented with 10µM Y27632. (D) Kidney cultured in CKCM supplemented with 20µM Y27632. Kidneys were fixed after 48hrs using ice cold methanol. Kidneys were stained for with mouse anti-Calbindin D-28K and detected using anti-mouse-FITC.

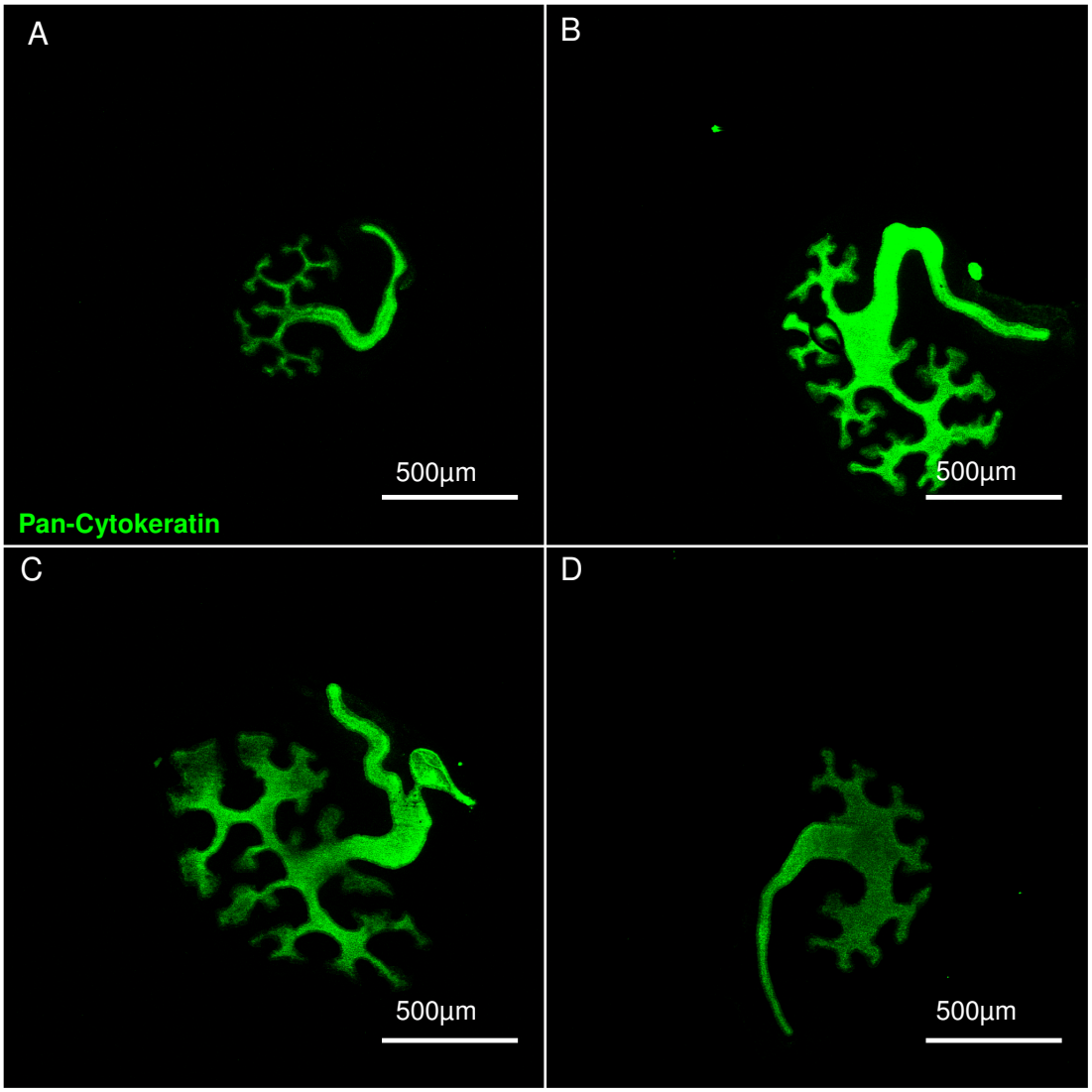


Fig.4.2 An additional ROCK inhibitor produces mimicking branching morphogenesis defects.

Fig.4.2 shows E11.5 kidneys that were dissected and cultured for 72hrs. Varying concentrations of H1152 Rho-Kinase inhibitor were used. (A) Kidney cultured in CKCM. (B) Kidney cultured in CKCM supplemented with 0.625µM H1152. (C) Kidney culture in CKCM supplemented with 1.25µM H1152. (D) Kidney cultured in CKCM supplemented with 2.5µM H1152. Kidneys were fixed after 72hrs using ice cold methanol. Kidneys were stained with mouse anti-pan-cytokeratin and detected using anti-mouse-FITC.

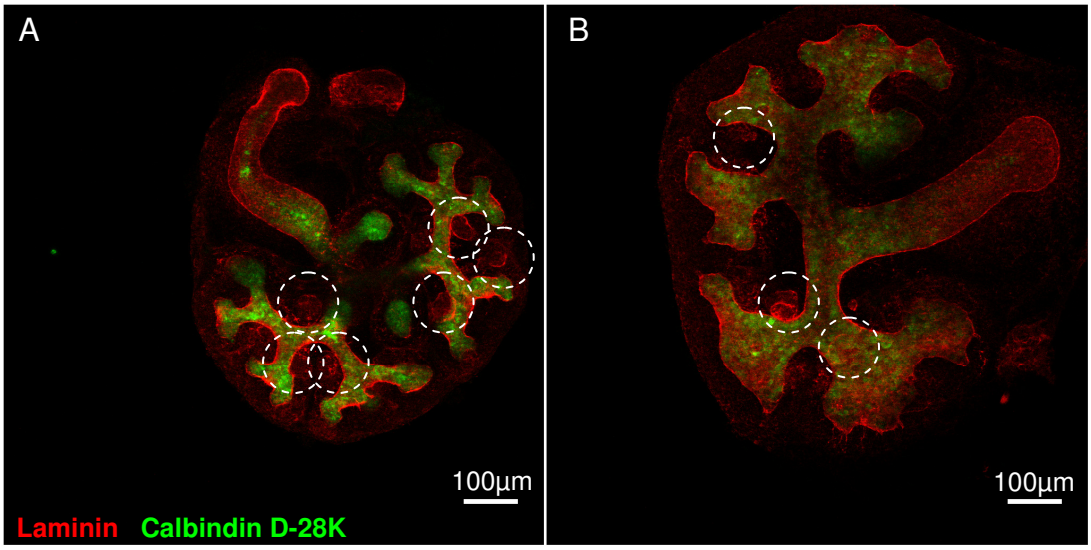
4.2.3 Rho-kinase inhibition reduced nephron formation.

The role of ROCK during nephrogenesis has not been investigated. Michael, Sweeney and Davies, and Meyer and colleagues suggested that nephrons do form normally in the presence of ROCK inhibitor, although their development was not studied in detail (Michael, Sweeney, and Davies 2005; Meyer et al 2006). My main aim was to determine whether ROCK plays a role during nephrogenesis. In Chapter 1, nephron development is described in some detail and some of the key genes are highlighted. From the introduction it is clear that although some of the inducers of nephron formation are emerging, the mechanisms of nephrogenesis remain unknown. In this chapter I outline the importance of ROCK for several different epithelial processes such as the regulation of polarity and adhesion. The remainder of this chapter focuses on the importance of ROCK during nephron formation and the subsequent stages of maturation which the nephron progresses through in order to become physiologically functional.

In order to visualise nephron development I used immunofluorescent staining for Calbindin D-28K and β-laminin to show both the ureteric bud and the basement membrane of the ureteric bud and the forming nephrons. Kidneys were cultured for 72hrs in control conditions, CKCM, (Fig.4.3A) (n=9 kidneys) or in CKCM supplemented with ROCK inhibitor (Fig.4.3B) (n=8 kidneys) prior to fixation and immunostaining. The number of nephrons in control conditions and in cultures with ROCK inhibitor was counted. The inhibition of ROCK reduced the number of nephrons that formed to only 54% of control numbers (Fig.4.3C). The reduction was shown to be statistically significant, $p=0.03$, using a one-tailed Student's t-test. This reduction in nephrons suggested that ROCK is important for the earliest stages of nephron formation. Possible reasons for this could be that ROCK is necessary for nephron induction or that ROCK is necessary for the mesenchymal-to-epithelial transition. β-laminin will only be present on nephrons that have become epithelial so stages earlier than the renal vesicle stage would not have been detected using this antibody.

Not only did the inhibition of ROCK result in significantly fewer nephrons but those nephrons that formed appeared morphologically abnormal, (Fig.4.3 and subsequent figures).

The differences between my results and those of Michael, Sweeney and Davies, and Meyer and colleagues (Michael, Sweeney, and Davies 2005; Meyer et al 2006) can be explained because of the differences of techniques that were used in order to study kidney development. The focus of previous reports has been on the role of ROCK for the branching morphogenesis of the ureteric bud (Michael, Sweeney, and Davies 2005; Meyer et al 2006). Because of this, the authors rarely utilised techniques that displayed both the nephrogenic components as well as the ureteric bud. In the majority cases, antibodies for Calbindin D-28K and lectin Dolichos biflorus were used, in combination with dark-field and phase-contrast microscopy (Michael, Sweeney, and Davies 2005; Meyer et al 2006). Calbindin D-28K and lectin Dolichos biflorus exclusively bind to the ureteric bud during early development and as such do not allow for the study of nephron development (Davies 1994; Michael, Sweeney, and Davies 2007). I on the other hand, specifically wished to determine whether ROCK is necessary for nephrogenesis and as such used appropriate markers for the purpose of visualising nephrons in addition to observing the nephrons closely.



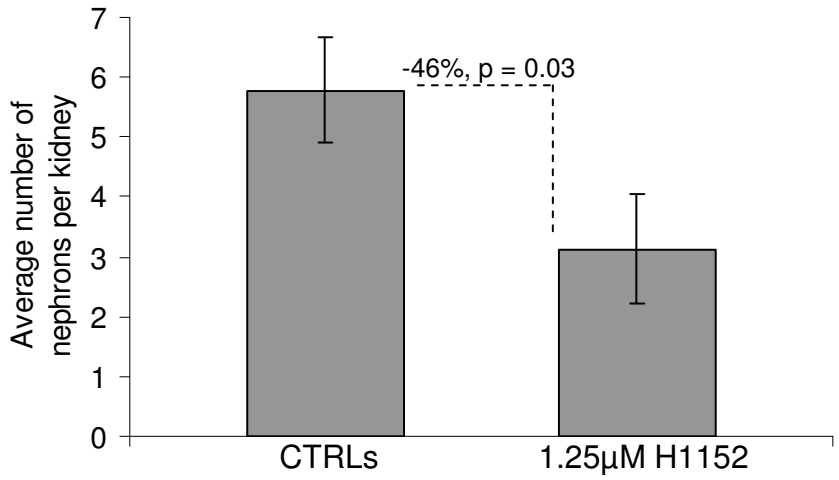


Fig.4.3 Inhibition of ROCK reduces nephron formation.

Fig.4.3 shows E.11.5 kidneys cultured for 72hrs in (A) CKCM or (B) 1.25µM H1152. Kidneys were fixed in ice cold methanol, stained for rabbit β-laminin and mouse calbindin D-28K and detected with anti-rabbit-TRITC and anti-mouse-FITC. White dashed-lined arrows indicate nephrons. (C) Bar chart showing the mean values ± SEM of the number of nephrons per kidney for each culture condition. Data was analysed separately using an unpaired one-tailed Student's t-test. T-test p-value p= 0.027, n =9&8 kidneys.

4.2.4 Rho-kinase inhibition disturbs normal nephron formation.

As previously described in Chapter 1, nephron development proceeds through several "descriptively termed" stages (Fig.1.1). It is possible to identify nephrons and stage them based on their morphology and it is also possible to recognise those nephrons that display developmental abnormalities. The nephrons forming in ROCK inhibitor conditions did not appear morphologically normal. I decided to investigate this further. Firstly, in order to determine the nature of the nephron abnormality, a higher detailed analysis was required. Secondly, it was necessary to determine whether any abnormalities detected were secondary effects of the abnormal ureteric bud. To provide a better background for any renal abnormalities, it is worthwhile to refer to (Fig.1.1) and (Fig.1.2) which show the normal development of a nephron in the human and a detailed diagram of an s-shaped mouse nephron, respectively.

Whole kidney cultures were set up in control conditions which generated nephrons in both the comma-shaped body (CB) and s-shaped body (SB) stages after 72 hrs (Fig.4.4A). Whole kidney cultures treated with inhibitors of ROCK showed nephrons which were considerably different (Fig.4.4C). These nephrons were either completely morphologically abnormal or abnormal towards their proximal parts, see (Fig.1.2) for a normal human nephron with labelled structures. In order to determine whether the morphological abnormalities were just an indirect result of the known effects that the ROCK inhibition has on the ureteric bud (Michael, Sweeney, and Davies 2005), I set up cultures of isolated metanephric mesenchyme that were induced to form morphologically organotypical nephrons by signalling, transfilter, from spinal cord (Saxen 1987). After 72 hrs of culture, control spinal cord induction cultures contained nephrons that were at either comma-shape body (CB) stage or at s-shape body (SB) stage (Fig.4.4B). Many nephrons grown under ROCK inhibiting conditions exhibited morphological abnormalities characterised by aberrant epithelial structures and cyst formation although some resembled normal nephrons (Fig.4.4D). These results indicate that ROCK is necessary for normal nephron formation and that abnormal nephrogenesis

is not an effect secondary to inhibiting ROCK in the ureteric bud. I also demonstrated the effects of ROCK inhibition on nephron formation using the metanephric mesenchyme from a *WT1*$^{+/GFP}$ mouse, (kindly supplied by Peter Hohenstein-HGU/MRC, Edinburgh) as is shown in (Appendix 2-Fig.A2.2). This mouse expresses GFP under the WT1 promoter (Hohenstein, P. Personal Communication). These results demonstrate that the effects of ROCK inhibition on nephrogenesis are not strain-specific as the *WT1*$^{+/GFP}$ mouse is of strain C57BL/6.

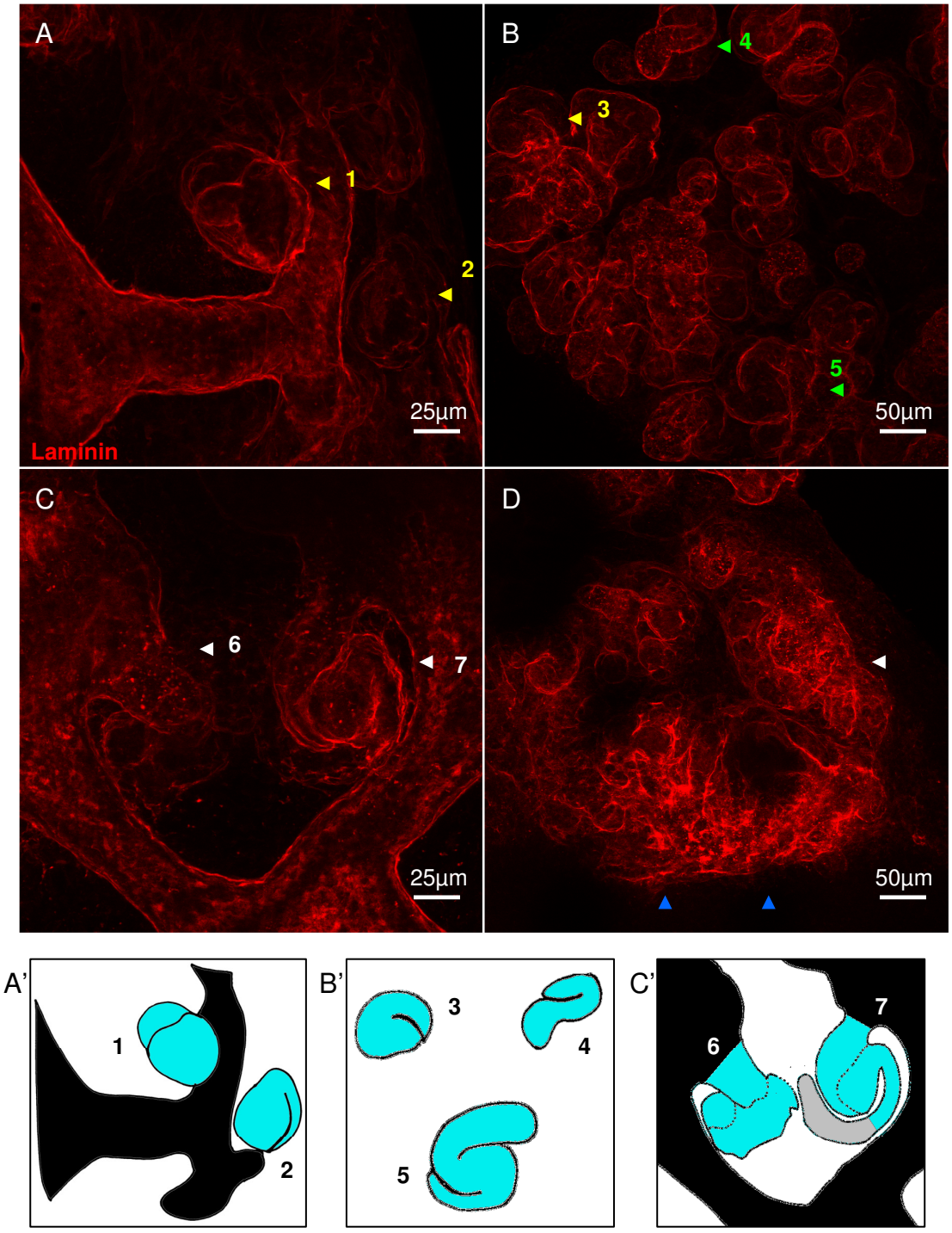


Fig.4.4 The effects of ROCK inhibition on nephrogenesis.

Fig.4.4 shows immunostaining of nephrons formed in control conditions or in the presence of ROCK inhibitor. The morphology of nephrons formed in CKCM (A,B) was compared with that of nephrons formed in CKCM with 20μM Y-27632 (C,D). (A) and (C) show nephrons formed in kidney cultures whereas (B,D) show nephrons formed in spinal cord induced metanephric mesenchyme. All cultures were kept for 72hrs after which they were fixed and stained with rabbit anti-β-laminin-rabbit and anti-rabbit-TRITC. Yellow arrowheads - comma-shaped nephrons, green arrowheads - s-shaped nephrons, white arrows - abnormal nephrons and abnormal epithelialisation. (A'-C') show schematic representations of numbered nephrons in (A-C). Nephrons are in turquoise and the ureteric bud in black. Diagrams were produced by careful inspection of nephrons viewing them at the maximum image resolution.

4.2.5 ROCK is necessary for glomerular cleft formation but nephron-ureteric bud fusion is preserved.

I used confocal microscopy to analyse the structural malformations of the nephrons formed under ROCK inhibiting conditions in detail. The nephrons in previous experiments showed some indications that the proximal portions of the nephrons might be more affected than the distal portions. To further investigate this, I set up whole kidney cultures where I allowed the nephron development to progress further than in previous experiments. In order to provide increased resolution of the abnormalities, I used three markers: Pan-cytokeratin, which binds cytokeratins present in the ureteric bud only; β-laminin which marks the basement membrane of both the ureteric bud and the forming nephrons, and TO-PRO-3, a nuclear marker.

In whole kidney cultures, nephrons formed after 96hrs of culture clearly displayed structural components of the proximal parts of the nephron, see (Fig.1.2) for a labelled nephron. The nephrons showed glomerular clefts, apical-basal flattening of the parietal epithelium, the Bowman's lumen and the visceral epithelium which gives rise to the podocytes (Fig.4.5A,B). Nephrons forming in ROCK-inhibitor conditions formed as one or two cysts in very close proximity of each other (Fig.4.5C). The cyst-like nephrons had no defining proximal or distal segments. A second type of nephrons formed in ROCK-inhibiting conditions where the nephrons consisted of a single tubule attached to the ureteric bud (Fig.4.5C,D). These tubules lacked proximal structures such as a glomerular cleft. These nephrons maintained the ability to fuse successfully to, and form a continuous lumen with, the ureteric bud (Fig.4.5C,D), suggesting that distal segments may retain normal function. These results also demonstrated that, regardless of the morphological deformities of the ureteric bud, adequate bud-nephron interactions were maintained in order to allow fusion. This ureteric bud fusion is an interesting process which can be disrupted in for example *Six2* mutant mice (Self et al 2006). The main morphological defect that could be characterised was the lack of a glomerular cleft. As

discussed in Chapter1, the mechanisms that drive the formation of the glomerular cleft are unknown.

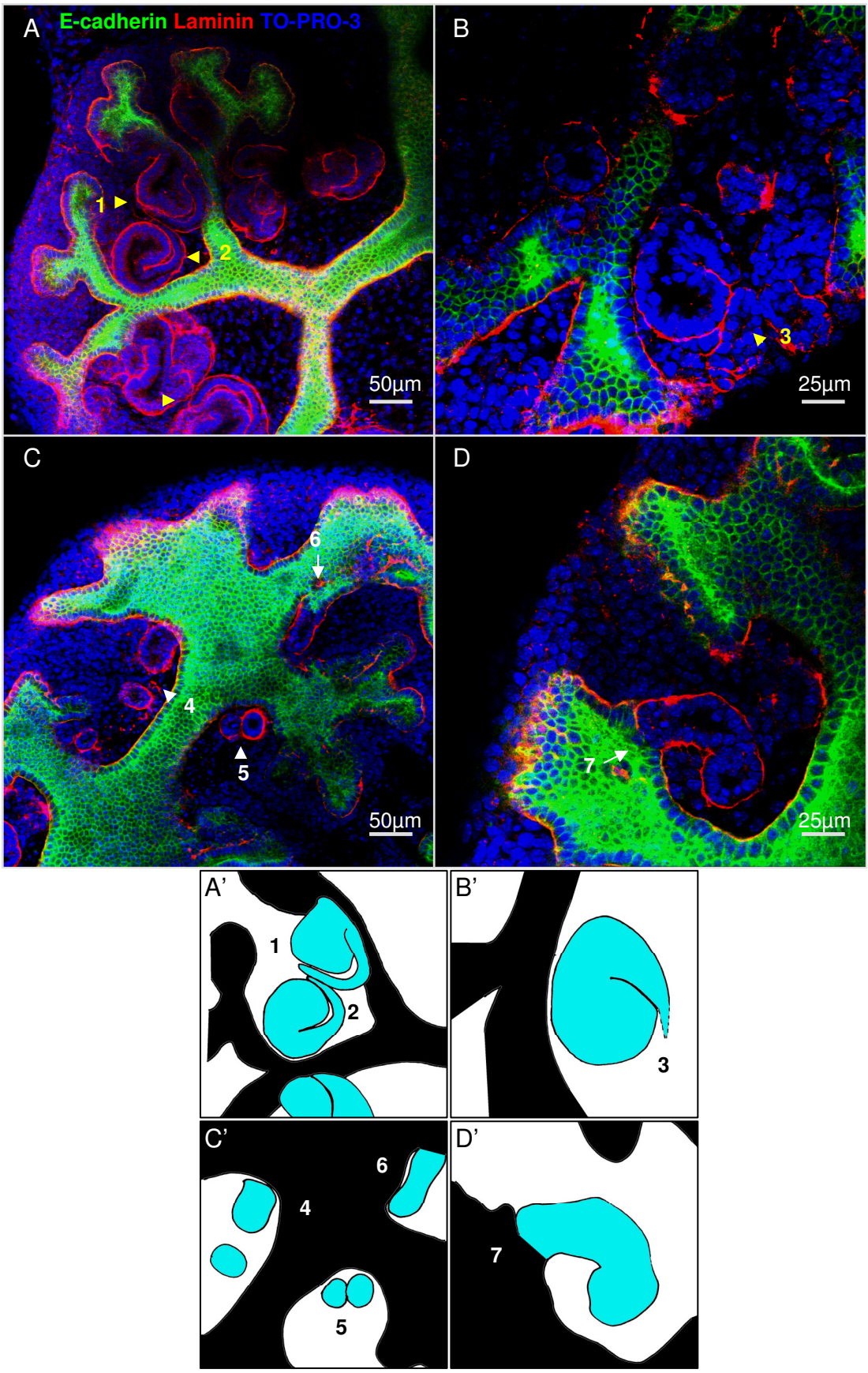

A
E-cadherin Laminin TO-PRO-3
1
2
50µm
B
3
25µm
C
6
4
5
50µm
D
7
25µm
A'
1
2
B'
3
C'
6
4
5
D'
7

Fig.4.5 The effects of ROCK inhibition on glomerular cleft formation and nephron morphology

Fig.4.5 shows immunostains of kidneys cultured for 96hrs. Images show nephrons formed in CKCM without (A and B) and with 1.25μM H1152 (C and D). Samples were fixed in methanol and stained with mouse anti-pan-cytokeratin, rabbit anti-laminin, anti-mouse-FITC, anti-rabbit-TRITC and TOPRO-3. Yellow arrowheads – nephrons with normal glomerular clefts, white arrowheads – abnormal nephrogenic cysts, white arrows – abnormal nephrons fused to the ureteric bud. (A'-D') show schematic representations of numbered nephrons in (A-D). Nephrons are in turquoise and the ureteric bud in black. Diagrams were produced by careful inspection of nephrons at maximum resolution.

4.2.6 ROCK is necessary for the nephron proximal-distal axis patterning.

The defects of the nephrons forming in the ROCK inhibitor conditions suggest that the proximal portions of the nephrons are negatively affected by the ROCK inhibition. To further characterise the nephron defects caused by inhibition of ROCK, and to test the possibility that the development of proximal and distal segments is affected differentially, I examined the expression of markers for proximal and distal portions of the nephron.

4.2.6.1 Rho-kinase inhibition disturbs distal nephron patterning.

E-cadherin has previously been shown to be present from the renal vesicle (RV) stage, the stage before the comma-shaped body (CB) stage, in the presumptive distal nephron and to be absent from the presumptive proximal nephron (Cho et al 1998). E-cadherin can therefore be used as an early marker for distalness and nephron proximal-distal polarity.

Whole kidney cultures were set up in either control or ROCK inhibiting conditions and cultured for 72hrs. Nephrons forming in whole kidney cultures grown in plain CKCM displayed a clear boundary between areas that were positive for E-cadherin and those that were negative (Fig.4.6A,C). The areas that were negative for E-cadherin included the proximal tubule and the parietal and visceral epithelia. Nephrons developing in ROCK-inhibiting conditions showed a more variable profile of E-cadherin localisation. A portion of nephrons that were clearly morphologically abnormal, as previously described, showed E-cadherin localisation throughout (Fig.4.6B,D). Some did, however, have normal E-cadherin localisation despite having abnormal proximal segments (Fig.4.6B).

These results show that a portion of the nephrons in ROCK inhibiting conditions display E-cadherin expression throughout the nephrons.

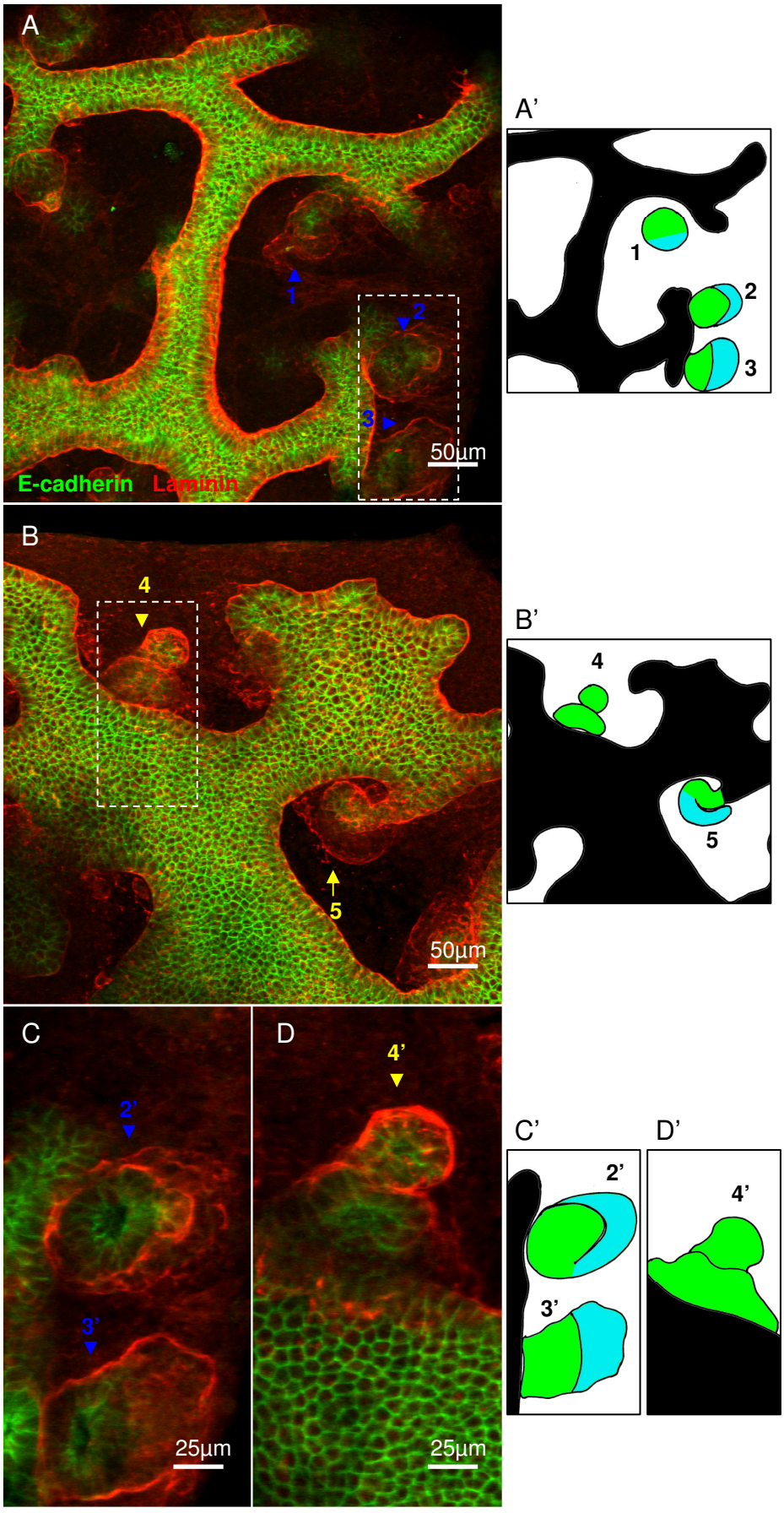

A
1
2
3
50µm
E-cadherin
Laminin
A'
1
2
3
B
4
5
50µm
B'
4
5
C
2'
3'
25µm
D
4'
25µm
C'
2'
3'
D'
4'

Fig.4.6 The effects of ROCK inhibition on nephron proximal-distal segmentation (I)

Fig.4.6 shows immunostainings of kidneys cultured for 72hrs. Images show nephrons formed in CKCM (A) and (C) and nephrons in 1.25μM H1152 (B) and (D). Samples were fixed in methanol and stained with mouse anti-E-cadherin and rabbit anti-β-laminin that were detected with anti-mouse-FITC and anti-rabbit-TRITC. (C) and (D) show highlighted regions in (A) and (B), respectively. Blue arrowheads – normal E-cadherin localisation in nephrons, yellow arrowheads – abnormal E-cadherin localisation in abnormal nephrons, - yellow arrow – normal E-cadherin localisation in abnormal nephron. (A'-D') show schematic representations of numbered nephrons in (A-D). Nephrons are in turquoise and the ureteric bud in black. Green segments of the nephron indicate parts positive for E-cadherin. Diagrams were produced by careful inspection of nephrons at maximum resolution.

4.2.6.2 Rho-kinase inhibition disturbs proximal nephron patterning.

I speculated that either of two mechanisms might account for the expression of E-cadherin throughout the nephron structure. Firstly, the proximal parts might have failed to form and only distal parts of the nephron formed. Secondly, proximal parts formed but failed to differentiate into the proximal phenotype and E-cadherin expression expanded into segments normally destined to be proximal. To test these hypotheses, I used markers for the proximal portions of the nephron. CD15 is normally detected in the proximal brush-border as the nephron matures (Bard and Ross 1991) and has previously been used as a marker for nephron maturation and proximal development (Davies 1994).

In control nephrons in whole kidney cultures cultured for 120hrs, CD15 was strongly detected in proximal tubules beyond the SB stage (Fig.4.7A). Inhibition of ROCK resulted in abnormal detection of CD15. CD15 was detected in nephrogenic cyst-like structures and in nephrons where CD15 was detected throughout (Fig.4.7B). In nephrons formed by spinal cord induction, control nephrons showed well organised CD15 localisation that was confined to one portion of the nephron (Fig.4.7C). ROCK inhibition resulted in the formation of non-tubular and short segments of tubular epithelia where CD15 was detected in patchy patterns (Fig.4.7D).

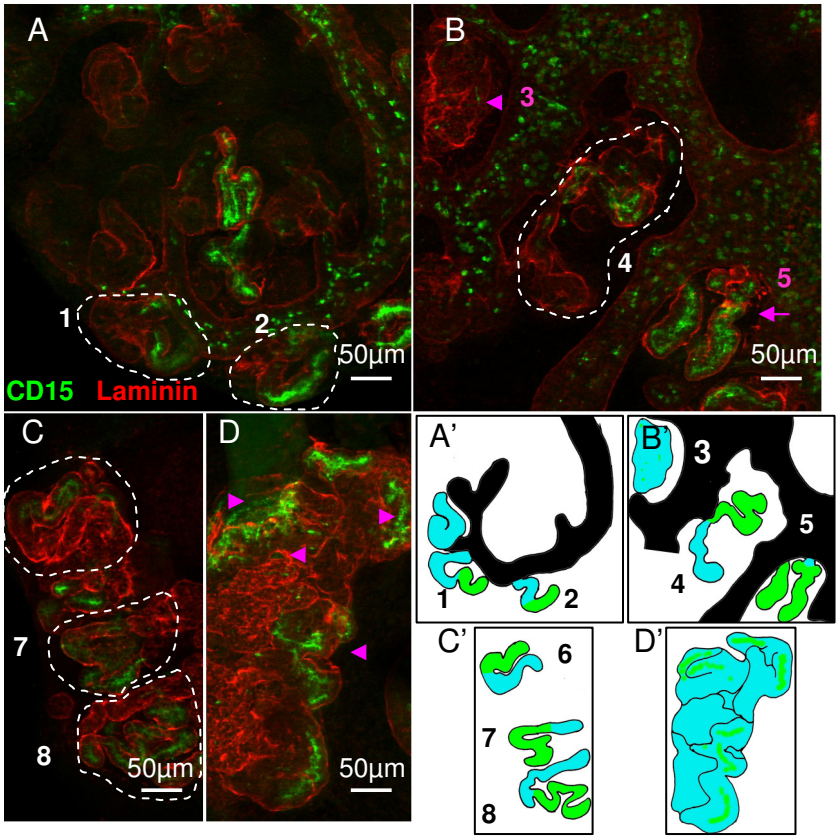


Fig.4.7 The effects of ROCK inhibition on nephron proximal-distal segmentation (#II)

Fig.4.7 shows immunostains of normal kidney cultures (A and B) and spinal cord induced MM (C and D). Controls were treated with CKCM (A and C) or with CKCM containing 20 µM Y-27632 (B and D). Cultures were maintained for 120hrs prior to fixation and subsequent staining with rabbit anti-β-laminin and mouse (IgM) anti-CD15 which were detected using anti-rabbit-TRITC and anti-mouse (IgM)-FITC. Magenta arrowheads – punctuated/abnormal CD15 localisation in nephrons, magenta arrows – nephrons with CD15 localised throughout, white dashed line – nephrons with normal CD15 localisation. (A'-D') show schematic representations of numbered nephrons in (A-D). Nephrons are in turquoise and the ureteric bud in black. Green segments of the nephron indicate parts positive for CD15. Diagrams were produced by careful inspection of nephrons at maximum resolution.

4.2.6.3 Rho-kinase inhibition disturbs proximal-distal patterning in the same nephron.

In the previous sections, two developmental defects have been described in the nephrons. A portion of nephrons has been shown where E-cadherin or CD15 is detected ectopically. In order to determine whether nephrons that expressed E-cadherin ectopically also mis-express proximal tubule marker CD15, and vice versa, cultures were set up for co-staining of the two markers.

To allow for nephron maturation and elongation of the proximal-distal axis, the kidney cultures were maintained for 144hrs. Samples were stained for mouse (IgG) anti-E-cadherin and mouse (IgM) anti-CD15, which were detected with anti-mouse (IgG) AlexaFluor 647nm and anti-mouse (IgM)-FITC. Nephrons that formed in control conditions expressed E-cadherin and CD15 in clearly defined distal and proximal regions, respectively (Fig.4.8A,C). CD15 and E-cadherin were found extensively in the same segments in ROCK inhibitor nephrons (Fig.4.8B,D). Control nephrons, demonstrated a high degree of apical-membrane polarised E-cadherin expression in distal segments (Fig.4.8C). At this stage of nephron development, capillary loop (CL) stage, E-cadherin was also weakly detected at the apical-membrane in proximal regions, although the expression was very diffuse. These differences in E-cadherin will be more clearly shown in (Fig.4.9).

These data clearly show that nephrons in ROCK inhibiting conditions ectopically express E-cadherin and CD15 in the same segments. This demonstrates that ROCK is necessary for normal nephron maturation along the proximal-distal axis.

An interesting effect of the ROCK inhibition is the ability of some nephrons to undergo multiple fusions to the ureteric bud (Fig.4.8B). This might indicate that the abnormal proximal-distal patterning disturbs the nephron's sense of orientation and regulation of ureteric bud fusion.

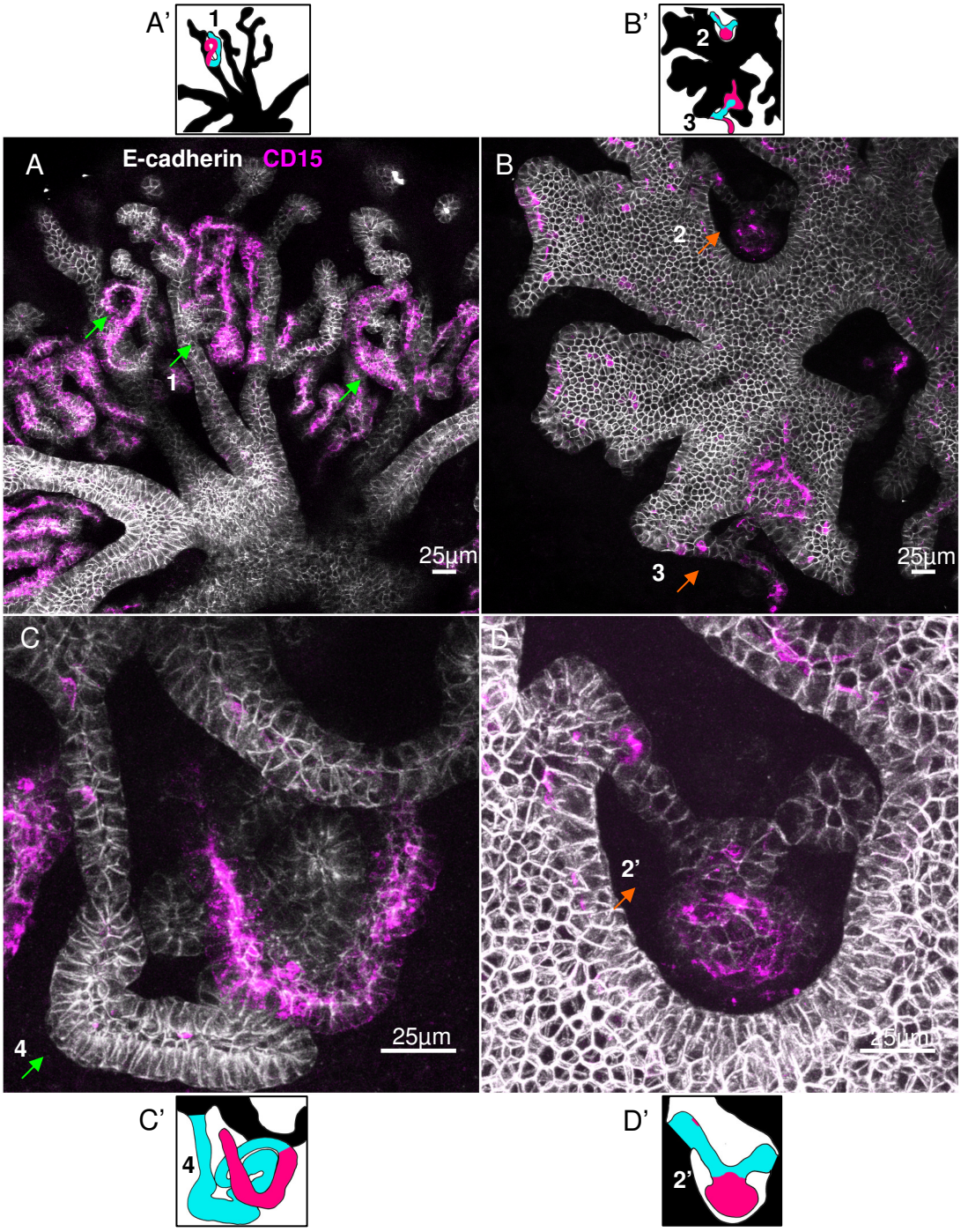


Fig.4.8 The effects of ROCK inhibition on nephron proximal-distal segmentation (#III)

Fig.4.8 shows whole kidney cultures maintained for 144hrs in control conditions (A, C) or in 1.25µM H1152 (B,D). Cultures were fixed in methanol and stained for mouse (IgG) anti-E-cadherin and mouse (IgM) anti-CD15 which were detected with anti-mouse (IgG)–AlexaFluor 647nm (displayed in grey) and

anti-mouse (IgM)-FTIC (displayed in magenta). The images are pseudocoloured as the AlexaFluor 647nm displays poorly on paper. In control nephrons, segments expressing high levels of E-cadherin are separate from those that express high levels of CD15 (A,C). Kidneys cultured in CKCM with ROCK inhibitor display nephrons that stably express both E-cadherin and CD15 in the same segments (B,D) and are capable of double-fusions to the ureteric bud. Green arrows – normal nephrons, orange arrows – abnormal nephrons. (A',B',C',D') show schematic representations of highlighted nephrons in (A,B,C,D). Nephrons are in turquoise and the ureteric bud in black. Magenta segments of the nephron indicate parts positive for CD15. Diagrams were produced by careful inspection of nephrons at maximum resolution.

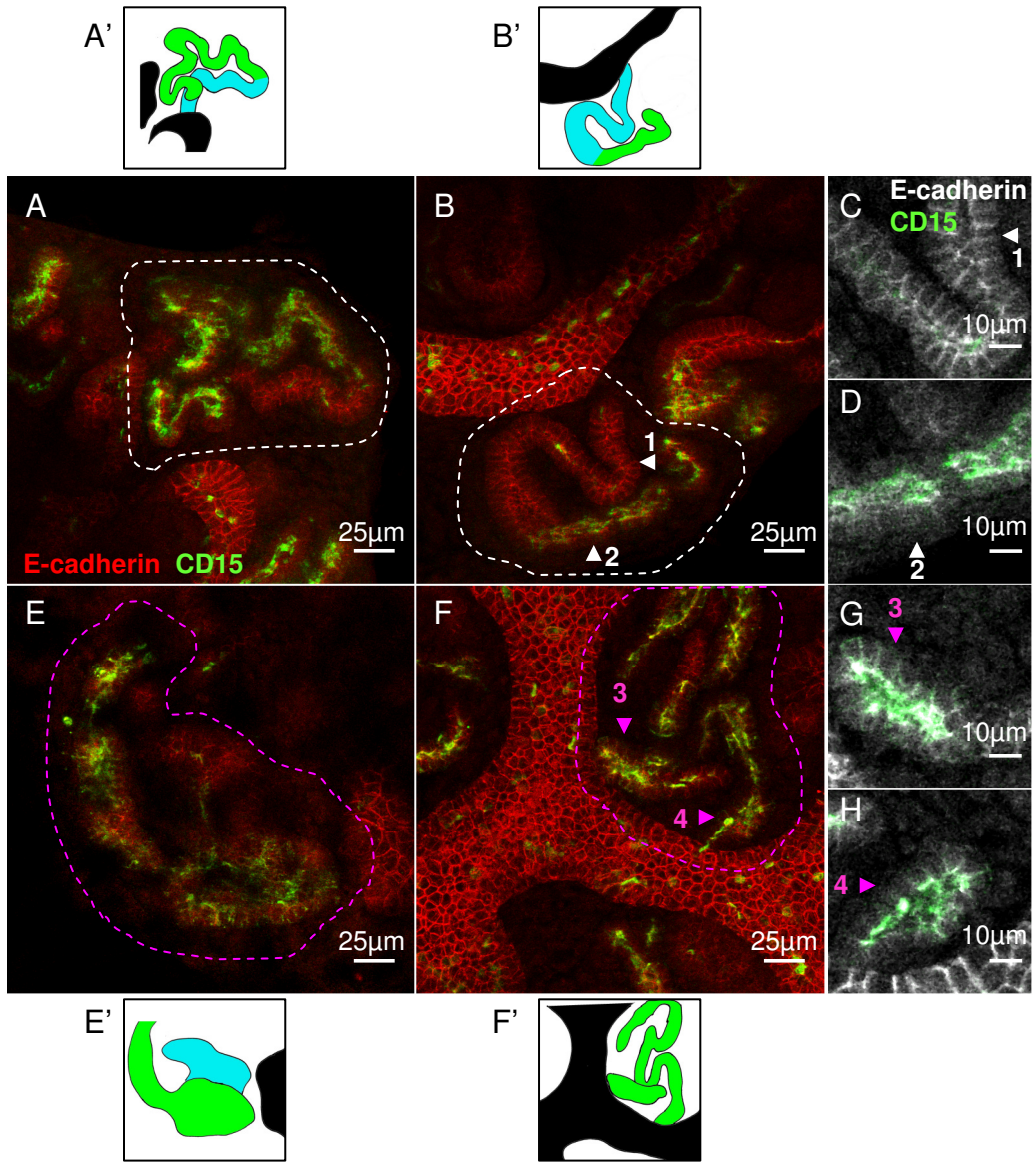
4.2.6.4 Rho-kinase inhibition disturbs proximal-distal patterning in the same nephron after renal vesicle formation.

ROCK is necessary for the patterning of the proximal-distal axis as shown using CD15 (proximal marker) and E-cadherin (distal marker). The experiments used to describe these effects yielded a relatively low number of nephrons since the inhibition of ROCK resulted in a large decrease in nephron formation; as shown in (Fig.4.3). Nephrogenesis is continuous and sequential, and new nephrons being constantly induced by the ureteric bud tips during development. In whole kidney cultures, where E11.5 kidneys are isolated and cultured, after 48hrs these samples contain nephrons that have progressed to renal vesicle (RV) and sometimes comma-shaped body (CB) stage. Since ROCK is necessary for nephron formation, one possible adjustment to the protocol that could produce a higher number of nephrons would be deferring the addition of ROCK inhibitor to after 48hrs of culture. This alteration would also show whether the nephrons are sufficiently polarised at the RV stage for them not to be affected by the inhibition of ROCK.

Whole kidney cultures were set up in control conditions and cultured for 48hrs. After 48hrs, half of the cultures were transferred to medium containing ROCK inhibitor and the other half simply remained in control conditions for an additional 72hrs. Concentrating on the proximal-distal border, control nephrons expressed E-cadherin and CD15 in clearly defined distal and proximal regions, respectively (Fig.4.9A,B). As described previously, nephrons at this advanced stage of maturity also expressed E-cadherin in the proximal segments although differently from in distal segments. Control nephrons demonstrated a high degree of apical-membrane polarised E-cadherin expression in distal segments, here magnified for clarity (Fig.4.9C). The E-cadherin that is weakly detected at the apical-membrane in proximal regions shows a very diffuse pattern (Fig.4.9D).

In ROCK inhibitor conditions, nephrons extensively show CD15 and E-cadherin in the same segments (Fig.4.9E,F). Large portions showed atypical CD15 and E-cadherin co-localisation (Fig.4.9G,H). Cells that displayed strong distal-like expression patterns of

E-cadherin also strongly expressed CD15 in a proximal-like manner (Fig.4.9G,H). These data clearly shows that the effects of ROCK are not just a consequence of the early developmental problem caused by ROCK inhibition. In fact, this demonstrates that the nephrons require ROCK for proximal-distal patterning subsequent to the RV stage. In the previous section, I showed that inhibition of ROCK resulted in some nephrons fusing to the ureteric bud at more than one place. This was likely to be a defect of proximal-distal segmentation because the ROCK inhibitor could be added after 48hrs (Fig.4.9I,J) or 72hrs (Fig.4.9K) with the same result as if the inhibitor was added at the start. The nephrons presented in this section display identical abnormalities to those presented in the previous section although nephrogenesis was much more prevalent. This made me opt for using this altered protocol for the subsequent experiments on proximal-distal polarity as a larger number of nephrons were available for analysis.


A'
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E-cadherin
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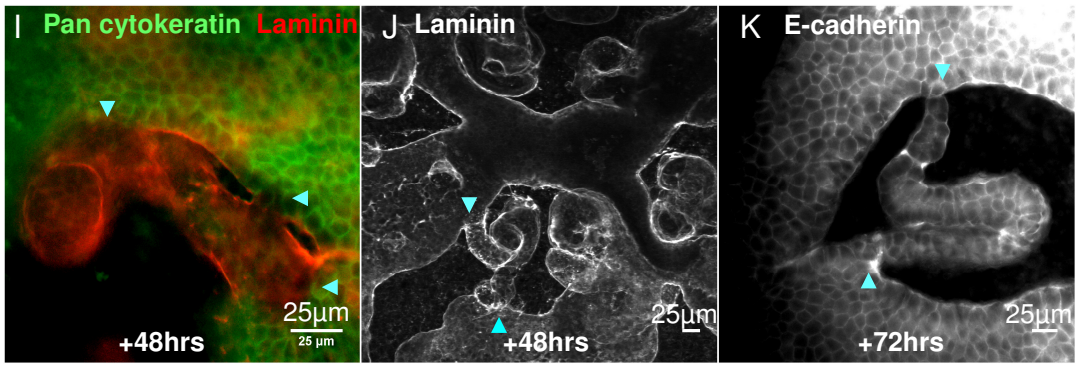

I Pan cytokeratin Laminin
J Laminin
K E-cadherin
25µm
+48hrs
25 µm
25µm
+48hrs
25µm
+72hrs

Fig.4.9 The effects of ROCK inhibition on nephron proximal-distal segmentation (#IV)

Fig.4.9 (A,B) Control nephrons cultured for 120hrs in CKCM show that segments with correctly patterned E-cadherin are mutually exclusive with those that express high levels of CD15, white dashed lines. (C,D) High magnification images of E-cadherin expression from (B), white arrowheads – no. 1-2, show distinctly different E-cadherin expression in distal segments where CD15 is absent (C) and in proximal segments where it its present (D). (E,F) Kidneys cultured for 48hrs in CKCM and then treated with ROCK inhibitor for 72hrs display nephrons that stably express both E-cadherin and CD15 in the same segments, magenta dashed lines. (G,H) High magnification images of E-cadherin expression from (F), magenta arrowheads – no. 3-4, reveal co-localisation of distal-like expression of E-cadherin and CD15. (A',B',E',F') show schematic representations of highlighted nephrons in (A,B,E,F) . Nephrons are in turquoise and the ureteric bud in black. Green segments of the nephron indicate parts positive for CD15. Diagrams were produced by careful inspection of nephrons at maximum resolution.
(I,J) and (K) Kidneys cultured for 48hrs or 72hrs in CKCM, respectively, prior to continued culturing in ROCK inhibitor for a total culture time of 144hrs. Turquoise arrowheads – nephron ureteric bud fusions.

4.2.6.5 Inhibition of ROCK disturbs proximal-distal patterning as shown using CD15 and WT1

The proximal-distal patterning defects shown in the previous sections could result from the disruption of two different mechanisms. Either the cells differentiate improperly and express markers for both proximal and distal segments or the cells differentiate appropriately but fail to segregate into the proper segments. The second mechanism could also result in abnormal differentiation. A disruption of cell segregation could result in cells being located in a signalling environment that induces ectopic gene expression and abnormal differentiation. In order to investigate the reason for the abnormal patterning, it is first necessary to show whether, on a single cell level, cells express markers for both proximal and distal segments or whether two cell types might be intimately mixed. It is also essential to demonstrate whether this mixing of segment fates can be show using different markers. To investigate these points I chose to use CD15 and WT1 as the markers. The expression pattern of CD15 has previously been described. In nephrons at this stage of maturity, WT1 is expressed in the podocytes, and weakly in the parietal cells. WT1 is clearly detected within the nucleus and in the cytoplasm (Hammes et al 2001).

Whole kidney cultures were set up as in the previous section where they were cultured for 48hrs and the transferred either to ROCK inhibitor conditions or maintained in control conditions. After 144hrs of culturing the samples were fixed and stained for mouse (IgG) anti-WT1, mouse (IgM) anti-CD15 and rabbit anti-β-laminin. These were detected with anti-mouse (IgG) AlexaFluor 647nm, anti-mouse (IgM)-FITC and anti-rabbit-TRITC. In control conditions, the nephrons showed well developed glomeruli, proximal tubules and distal tubules (Fig.4.10A). Nephrons that matured in ROCK inhibiting conditions showed abnormal glomeruli, where the glomeruli appears to be "burst" and not properly enclosed. Significantly, WT1 was detected in the CD15 positive tubule portions (Fig.4.10B). Co-localisation detection using ImageJ 1.40g, on single z-sections revealed that the WT1 stain co-localised with the CD15 in the tubular

portions of the nephron (Fig.4.10D). This did not occur in the control nephrons (Fig.4.10C).

These results show that there were cells in the tubular portion of the nephron which ectopically expressed WT1 in addition to expressing the endogenous marker proximal tubule marker CD15. This indicates that cells are displaying a defect in differentiation and that ROCK is necessary for normal nephron differentiation. This data suggest that close mixing of normally differentiated cell types was not the reason why different segment markers were detected in the same regions. The hypothesis which suggested that cells first failed to segregate and subsequently displayed erroneous differentiation is still plausible. Together with the data which showed E-cadherin and CD15 in the same cells, this strongly argues for the point that ROCK is required for the normal specification of cellular fates along the proximal-distal nephron axis.

I also attempted to use L1-cell adhesion molecule, a distal tubule specific protein (Debiec, Christensen, and Ronco 1998), as a marker. L1-cell adhesion molecule turned out to be unsuitable for this analysis because nephrons formed in control conditions displayed large regions of overlapping expression between L1-cell adhesion molecule and CD15. This suggests that L1-cell adhesion molecule is perhaps not as restricted to distal regions during early development as previously suggested (Debiec, Christensen, and Ronco 1998), although my results could be a result of the culture systems used in these experiments.

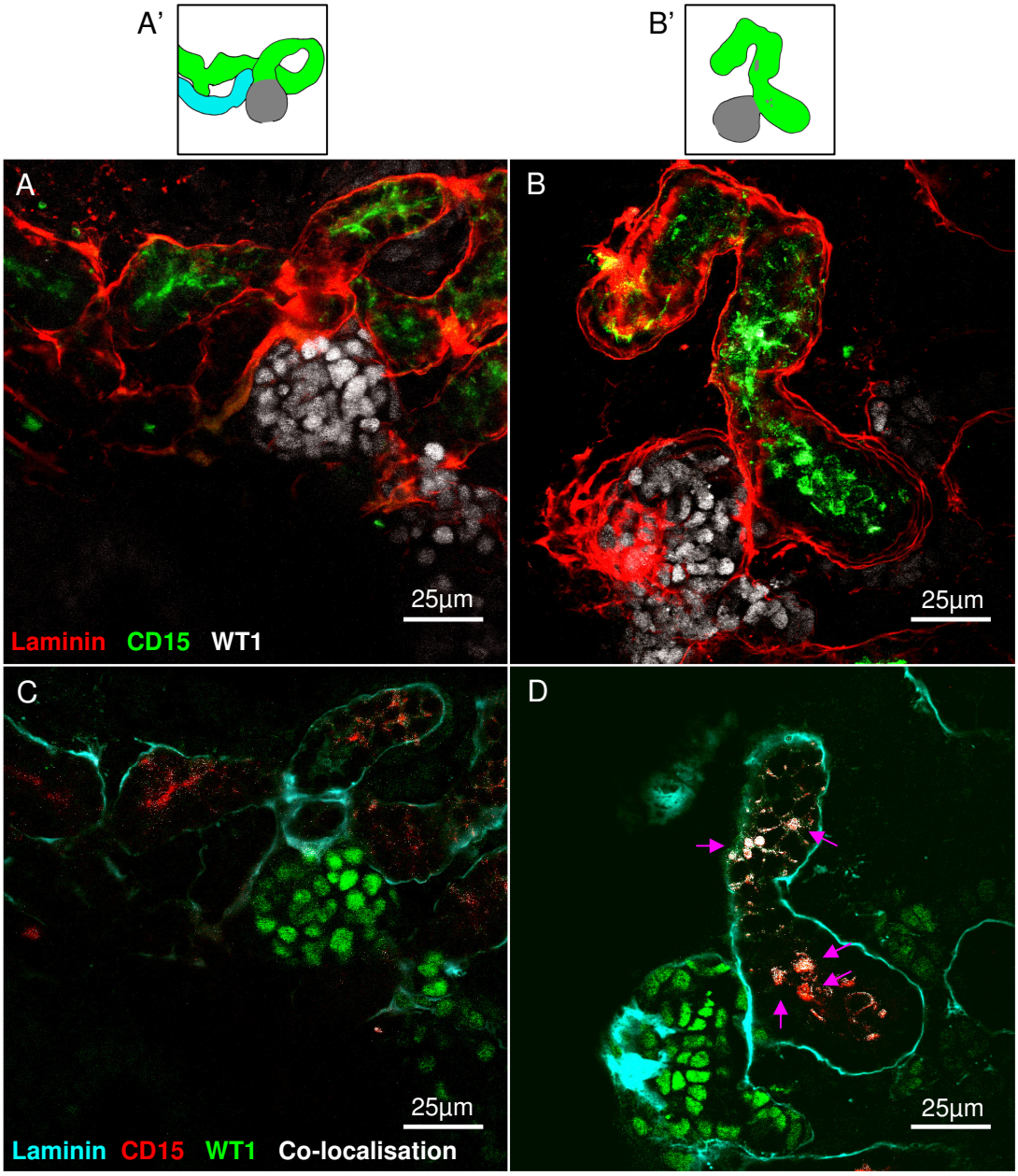


Fig.4.10 The effects of ROCK inhibition on nephron proximal-distal segmentation (#V)

Fig.4.10 (A) Nephron developed in control conditions for 144hrs show normal expression of CD15 in the proximal tubule, WT1 in the podocytes and β-laminin in the basement membrane. (B) Shows abnormal nephron developed in 1.25µM H1152 medium where the tubule portion is shortened and CD15 is present throughout. The glomerulus is abnormal as shown by the aberrant WT1 and β-laminin expression. (C,D) single z-sections of each nephron showing co-localisation of WT1 and CD15 as shown in white. (C) no co-localisation at all. (D) WT1 is detected in the CD15 expressing segment where co-localisation is

evident. The detected WT1 in (A,B) and WT1, CD15 and β-laminin in (C,D) are pseudocoloured for contrast or display purposes. Magenta arrows – points of co-localisation. (A',B') show schematic representations of nephrons in (A,B). Nephrons are in turquoise, green and grey segments of the nephron indicate parts positive for CD15 and WT1, respectively. Diagrams were produced by careful inspection of nephrons at maximum resolution.

4.2.7 Inhibition of ROCK does not prevent expression of nephron segment-specific genes.

In the previous sections, it was shown that ROCK is necessary for normal proximal-distal nephron patterning. This data suggested that the inhibition of ROCK affects normal differentiation and can directly, or by a secondary mechanism, affect gene expression. A noteworthy observation was that in spite of ROCK inhibition disturbing proximal-distal differentiation, this did not lead to an actual loss in the number of segment markers present; at least not in terms of L1-cell adhesion molecule, WT1, E-cadherin and CD15. This might suggest that ROCK does not regulate gene expression, but as suggested, ROCK could be part of the structural mechanism that produces the proximal-distal axis.

To investigate this point I decided to monitor the effects of ROCK inhibition on the expression of genes that have been shown to have an expression pattern restricted to either the presumptive Proximal Tubule, Loop of Henle, or the Distal Tubule. A large number of genes have been shown to display expression patterns that are specific to particular nephron segments (Raciti et al 2008). The purpose of this investigation was to determine whether the inhibition of ROCK might genetically affects the induction of nephron segmentation or if the inhibition of ROCK primarily affects a particular portion of the nephron more so than the other segments. The genes that were selected were chosen because they have highly restricted expression patterns. The proximal segment markers were: *sodium glucose transporter 2/solute carrier family 5 member 2 (sglt2/slc5a2)*, *hairy/enhancer-of-split related with YRPW motif 1 (hey1)* and *cadherin-6 (cdh6)* since they have been demonstrated to be expressed in the proximal portions of the nephron with no expression in distal parts or in other kidney structures (Cho et al 1998; Rubera et al 2004; Chen and Al Awqati 2005). Similarly, *iroquois related homeobox 3 (irx3)* and *hairy and enhancer of split 5 (hes5)* were picked as Loop of Henle markers, as they are not expressed in the more proximal or distal potions of the nephron or elsewhere in the kidney (Piscione, Wu, and Quaggin 2004; Reggiani et al

2007). B*rn1/Pou3f3*, *Tamm-Horsfall protein (THP)* and *hormonally upregulated neu-associated kinase/mak-v (hunk/mak-v)* were used as markers for the distal segments of the nephron for the same reasons as have been stated for the proximal and Loop of Henle markers (Nakai et al 2003; Sakai et al 2007).

Cultures were set up to monitor the expression of genes at several time-points of nephron maturation (24 hrs, 48 hrs, 72 hrs, 96 hrs and 144 hrs) (Fig.4.11). As in the previous sections, the ROCK inhibitor was added after 48hrs to allow for the formation of renal vesicles (RV). This meant that for time-points '24hrs' and '48hrs', these samples had only been cultured in control conditions. For time-points '72hrs', '96hrs' and '144hrs', these contained separate sets of kidneys that had been cultured from the 48hrs point in either control or ROCK inhibitor conditions. Multiple cultures were set up for each condition and cultured separately. RNA was isolated from the cultured kidneys at the specified time-points and represents RNA from 7-10 kidneys per condition and time. Protocols for RNA isolation, cDNA synthesis, primer design and PCRs are as described in Chapter 2. β-actin was used as a cDNA loading protocol. All original gel-images used to produce (Fig.4.11) are shown and labelled in Appendix 2-Fig.A2.3.

Expression of proximal tubule markers *sglt2* (Rubera et al 2004), *hey1* (Chen and Al Awqati 2005) and *cdh6* (Cho et al 1998), was similar in kidneys cultured in control and in ROCK inhibiting conditions. However, the expression of the segment markers was slightly lower in ROCK inhibitor cultures compared to control cultures. The same trend was seen when Loop of Henle markers *irx3* (Reggiani et al 2007) and *hes5* (Piscione, Wu, and Quaggin 2004) were analysed. *Hes5* was never detected in ROCK inhibitor cultures; however it was only weakly detected at 144hrs in control condition cultures and may have appeared if the cultures could have been maintained for a longer time. Whole kidney cultures maintained for more than 144hrs appeared non-viable. Distal tubule markers *brn1* (Nakai et al 2003), *THP* and *hunk* (Sakai et al 2007) showed the same tendency as previously discussed genes. Each gene specified is accompanied by the relevant reference specifying its restrictive expression pattern. These results indicate

that the inhibition of ROCK did not severely prevent gene expression during nephron maturation. Since the ROCK-inhibiting cultures displayed reduced nephron formation compared to controls, this may account for the reduced overall presence of nephron gene mRNA.

Time (days) and culture conditions control (C) & ROCK inhibitor (RI)

	GENE	24hrs C	48hrs C	72hrs C	72hrs RI	96hrs C	96hrs RI	144hrs C	144hrs RI
Proximal Tubule	SGLT2	-	-	(+)	-	(+)	(+)	+	(+)
	Hey1	+	+	+	(+)	+	(+)	+	+
	Cdh-6	+	+	+	(+)	+	+	+	+
Loop of Henle	Irx3	+	+	+	(+)	+	(+)	+	+
	Hes5	-	-	-	-	-	-	+	-
Distal Tubule	Brn1	+	+	+	(+)	+	+	+	+
	THP	-	-	-	-	-	-	+	(+)
	Hunk	+	+	+	(+)	+	(+)	+	+

Fig.4.11 Inhibition of ROCK does not disturb nephron segment-specific gene expression

Fig.4.11 shows the expression profile of kidneys that were cultured in CKCM for the first 48hrs and then further cultured in either CKCM (C) or 1.25µM H1152 ROCK inhibitor (H) until 144hrs. RNA was isolated at time-points: 24hrs, 48hrs, 72hrs, 96hrs and 144hrs. Gene expression was detected using end-point PCRs. The presence or the absence of particular gene transcripts at each time point and under each condition is scored as; '+' where the transcript was abundant, '-' where it was not detected or '(+)' where detection was very low. Genes known to display restricted and specific expression to Proximal Tubule (maroon), Loop of Henle (navy blue) and the Distal Tubule (purple) were chosen to in order to monitor nephron-segment differentiation and maturation. The expression of each gene in each condition is read across the table. Differential expression where transcripts were not detected or were only very weakly detected have been circled in red. The original gel-images are included in Appendix 2-Fig.A2.3 where the corresponding bands have been similarly marked.

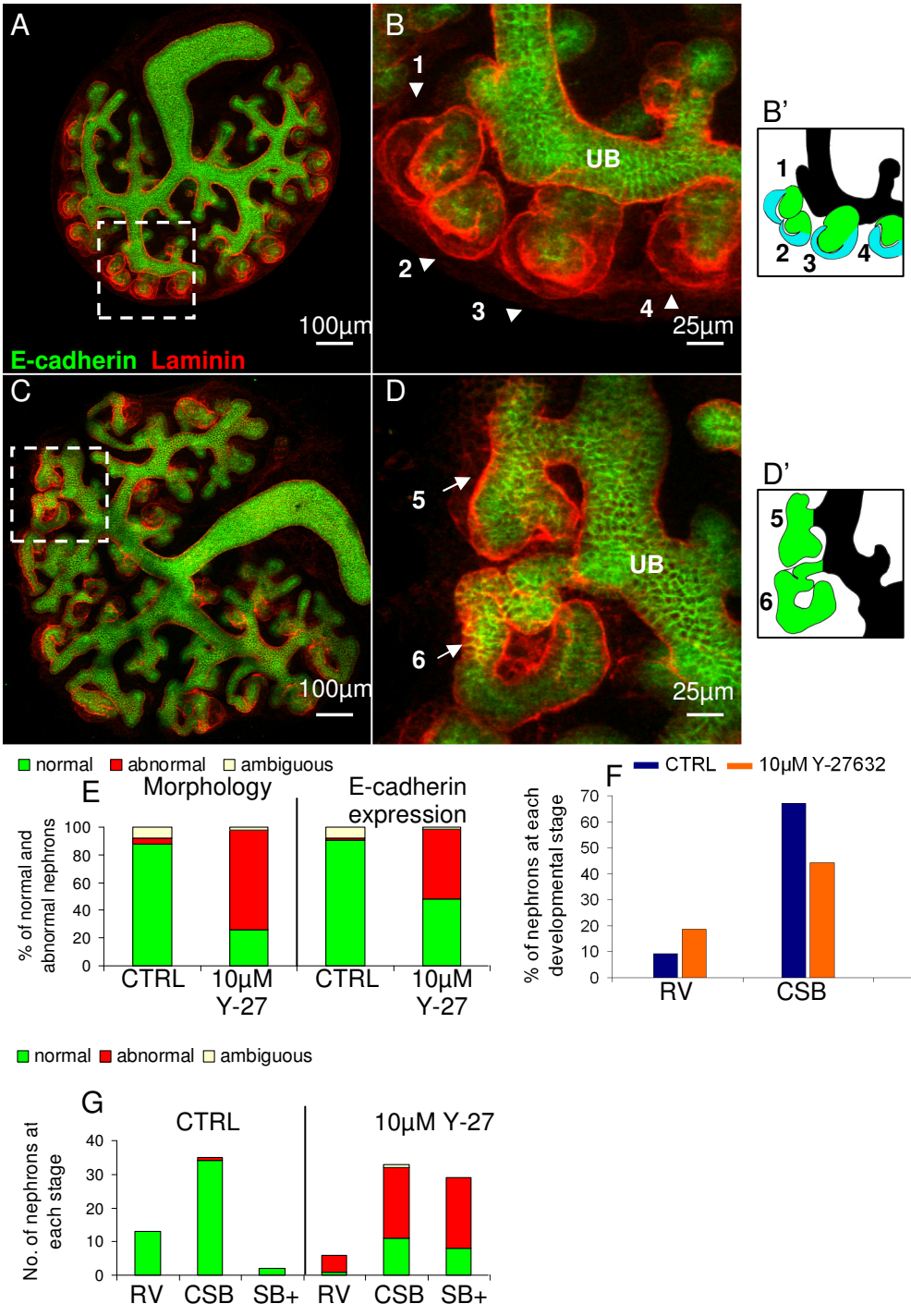
4.2.8 Inhibition of ROCK disturbs normal nephron maturation and in particular renal vesicle formation.

Inhibition of ROCK resulted in abnormal proximal-distal axis patterning but at the same time, nephron cells displayed E-cadherin, CD15, L1-cell adhesion molecule and WT1 protein and kidneys cultured in ROCK inhibitor conditions were shown to be expressing 7 proximal-distal segment specific genes. Although the abnormal proximal-distal patterning might account for the failed glomerular cleft formation seen in ROCK-inhibitor nephrons, I decided to further elucidate the importance of ROCK for both nephron formation and maturation by quantifying the degree and type of abnormality on a background of specific nephron stages. The aim was to determine critically the major stage of nephron formation affected by ROCK inhibition. The nephrons in control and ROCK-inhibiting conditions were scored using their morphology, E-cadherin localisation and stage. This enabled accurate assignation of nephrons into three stage groups: (1) Renal Vesicles (RV), consisting of simple cyst-like nephrons, (2) Comma- and S-shaped Body (CSB), nephrons that displayed a condensed tubular structure with a clearly visible glomerular cleft, and (3) S-shaped Body + (SB+), nephrons that showed an expansive tubular morphology.

Whole kidney cultures were set up as described in sections 4.2.6.4 - 4.2.7 where the after 48hrs of culture the ROCK inhibitor was added to the experimental samples and the kidneys maintained for a total of 120hrs. The cultures were fixed and stained for β-laminin and E-cadherin. 136 nephrons were examined of which 58 were nephrons from control kidneys and 78 were nephrons formed in ROCK inhibitor kidneys. In control cultures, nephrons displayed clearly restricted E-cadherin expression (Fig.4.12A,B) compared to ROCK inhibition cultures where E-cadherin expression was expanded into the whole of the nephrons (Fig.4.12C,D). In control cultures, nephrons of categories RV, CSB and SB+ stages contained 0%, 3% and 0% of nephrons which were classified as abnormal. A small proportion of control nephrons displayed ambiguous localisation of

E-cadherin and/or morphology and could not be classified with certainty as neither normal nor abnormal (Fig.4.12E).

Contrary to the control nephrons, 71% of ROCK inhibitor nephrons displayed abnormal morphology and 50% had abnormal localisation of E-cadherin (Fig.4.12E). The majority of nephrons formed in ROCK inhibiting conditions were in CSB or SB+ stages, 43% and 39%, respectively (Fig.4.12F). However, the proportionally larger number of nephrons at SB+ stage may have been a result of the morphological defects that these nephrons showed. A large number of SB+ nephrons were simply long tubules with no signs of proximal segments but with a more tubular morphology than what would be classified as a CB or SB nephron. The abnormal morphology and aberrant localisation of E-cadherin was seen in all stages of ROCK inhibitor nephrons but, proportionally, the greatest number of abnormal nephrons was seen at RV stage where 83% of nephrons were abnormal compared to CSB, 66%, and SB+, 72% (Fig.4.12G). These results show that nephrons forming in ROCK-inhibiting conditions develop anomalously and display segmental and morphological defects at several developmental stages. The relatively high proportion of abnormal renal vesicles presents the possibility that ROCK activity is of particular need during renal vesicle formation and maturation into CB stage.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D'
normal
abnormal
ambiguous
E
Morphology
E-cadherin expression
% of normal and abnormal nephrons
CTRL
10µM Y-27
F
CTRL
10µM Y-27632
% of nephrons at each developmental stage
RV
CSB
SB+
G
No. of nephrons at each stage

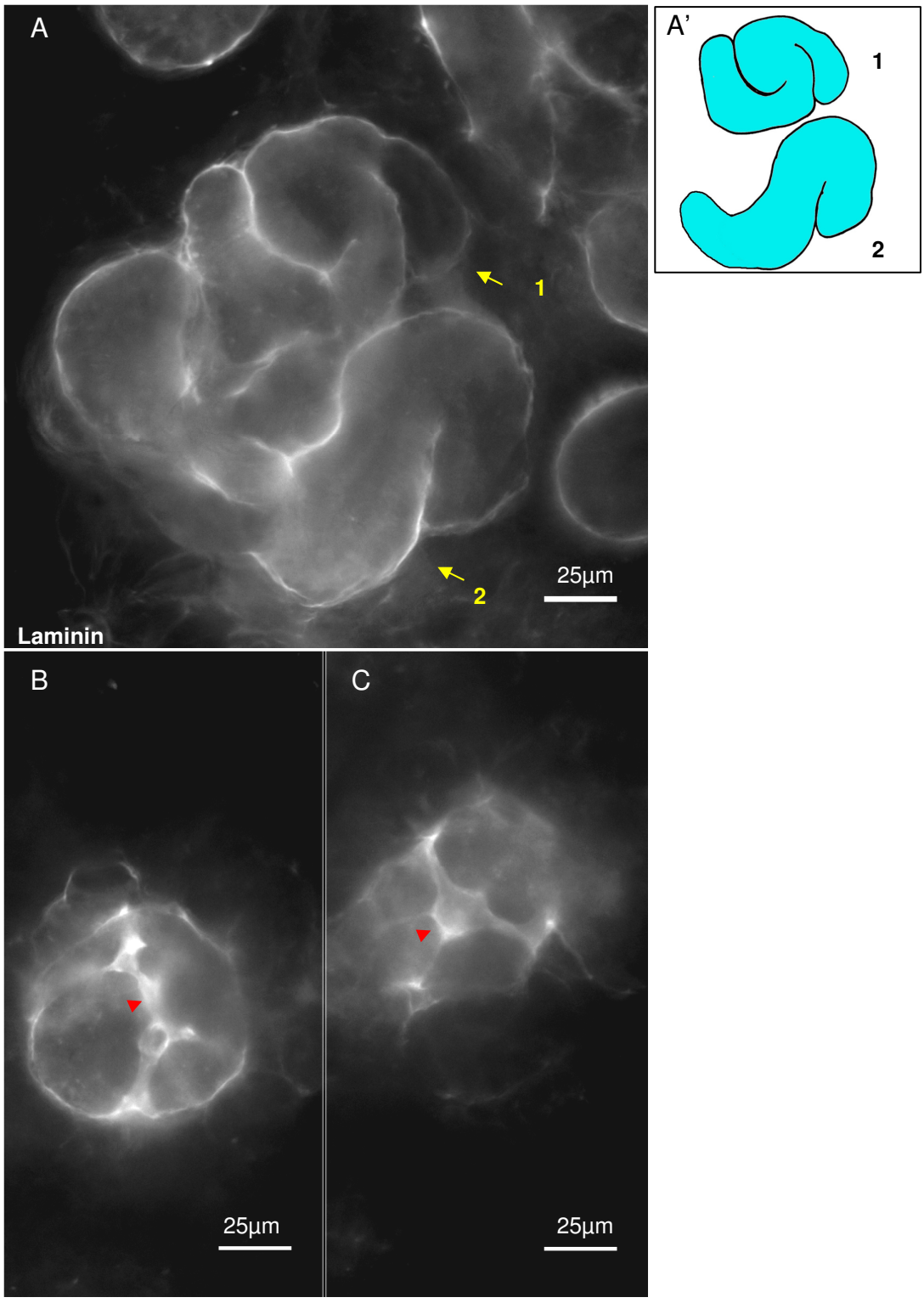
Fig.4.12 Quantitative analysis of ROCK effects on nephron maturation.

Fig.4.12, (A,B) Confocal images of E-cadherin and laminin immunostaining illustrate morphology and protein localisation in control nephrons, white arrowheads – nephrons 1-4, after 120 hrs of culture. (C,D) Nephrons where ROCK inhibitor was added after 48hrs of culture show nephrons that display abnormalities in morphology and/or E-cadherin expression, white arrows – nephrons 5-6. UB – ureteric bud. (B',D') show schematic representations of numbered nephrons in (B,D). Nephrons are in turquoise and the UB in black. Green segments of the nephron indicate parts positive for E-cadherin. Diagrams were produced by careful inspection of nephrons at maximum resolution. (E) Quantitative analysis of nephrons show that 71% of all nephrons in the ROCK inhibitor cultures exhibited abnormal morphology and 50% had abnormal E-cadherin expression. (F) Nephrons were also scored for their developmental stage; Renal Vesicle (RV) stage, Comma and S-shaped body stage (CSB) or S-shaped body + (SB+). Inhibition of ROCK results in a slight increase of nephrons that were SB+ stage compared to controls. (G) Inhibition of ROCK resulted in proportionally similar numbers of abnormal nephrons in CSB and SB+ stage whereas most nephrons in RV were classified as abnormal. 58-CKCM nephrons and 78-ROCK inhibitor nephrons were analysed.

4.2.9 ROCK is necessary for apicobasal polarity formation during early nephrogenesis.

To establish whether the reduced nephron formation and the high proportion of abnormal renal vesicles in ROCK inhibiting cultures could be a result of early nephrogenic defects, I decided to investigate the first structurally defined stages of the epithelial nephron. As a nephron forms from the mesenchymal-to-epithelial transition of the cells that surround and receive inductive signals from the ureteric bud tip, it is necessary for these epithelialising cells to develop apical, basal and lateral polarities in the process of becoming epithelial. Normal nephron cells possess an apical, luminal, side and a basal, admesenchymal, surface. Previous studies, displayed here as (Fig.4.13D,E) with kind permission from Dr Lipschutz, have shown Rho and ROCK to play an important role for apicobasal polarity formation in Madin-Darby Canine Kidney (MDCK) cell cysts that are cultured in collagen gels (Rogers et al 2003; Yu et al 2008). MDCK cells expressing a dominant-negative form of RhoA show inverted polarity with the apical surface facing the outside (Rogers et al 2003). One possibility is that the morphologically abnormal nephrons in my experiments may have formed as a result of the epithelialising cell's experiencing problems with properly defining their apicobasal axis during initial steps of the mesenchymal-to-epithelial transition.

To investigate whether apicobasal polarity formation occurred normally, spinal cord induction cultures were set up. In plain CKCM, nephron cells formed with normal polarity, the basal marker laminin being to the admesenchymal side of the epithelium and within the glomerular cleft (Fig.4.13A). In ROCK inhibitor cultures, the nephron cysts that formed in the spinal cord induction cultures displayed laminin on the luminal side of the epithelial cysts and not on the admesenchymal side (Fig.4.13B,C). These nephrons resembled the inverted MDCK cell cysts published by Rogers and colleagues where actin was used as a polarity marker (Rogers et al 2003). In whole kidney cultures, the concentration of ROCK inhibitor used prevented nephron formation but laminin deposition occurred between ureteric bud tips; compare controls (Fig.4.13F) with experiments (Fig.4.13.G).


A
A'
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2
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2
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Laminin
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C
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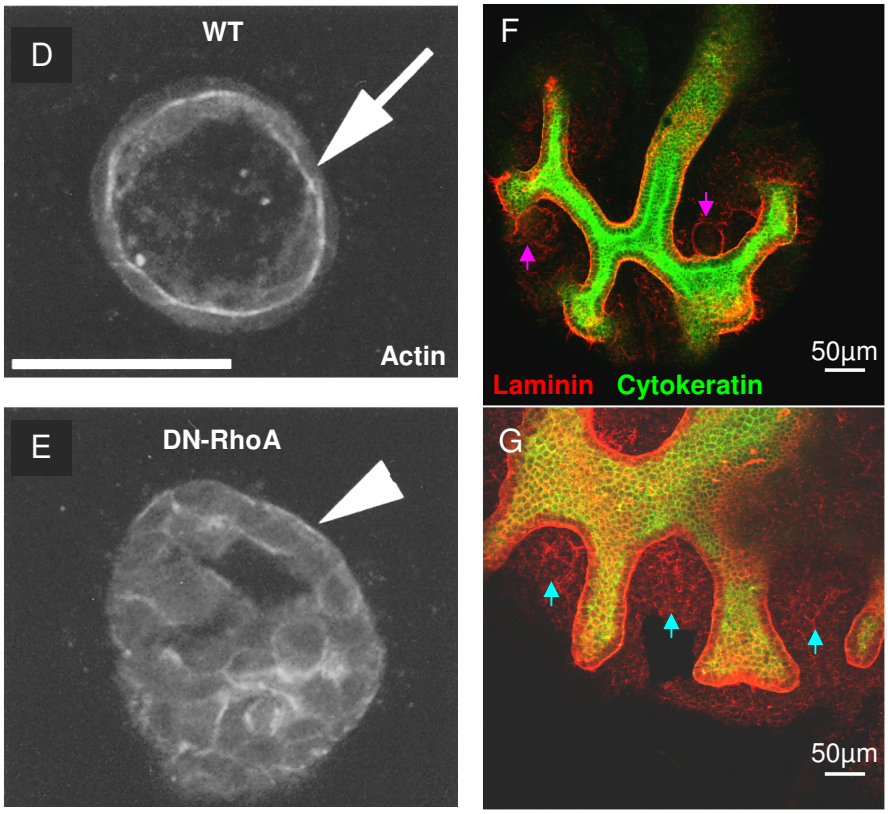


Fig.4.13 Inhibition of ROCK disturbs early apical-basal polarity formation in the renal vesicle

Fig.4.13 Shows immunostaining visualising the effects of ROCK inhibition on early stages of nephrogenesis. Transfilter spinal cord induced MM cultures were kept for 72hrs with (A) or without (B and C) 2.5µM H1152-glycyl. Cultures were stained for laminin using anti-laminin-rabbit and anti-rabbit-TRITC. S-shaped bodies are indicated with yellow arrows. Abnormal laminin distribution is specified with red arrow heads. (D) and (E) have been taken and modified, with kind permission of Dr Lipschutz, from Rogers et al 2003. Original figure annotations for (D) and (E) were Fig. 2 B and F, respectively. (D) and (E) display Madin-Darby canine kidney cell cysts stained for actin using phalloidin. Normal apical actin staining is indicated by the white arrow and actin staining indicating abnormal polarity is pointed out with white arrowheads. (F,G) Whole kidney cultures kept for 72hrs in either plain CKCM or in 2.5µM H1152, respectively. These kidneys were stained for rabbit β-laminin and mouse Pan-cytokeratin that were detected with anti-rabbit-TRITC and anti-mouse FITC. Normal nephrons are specified with magenta

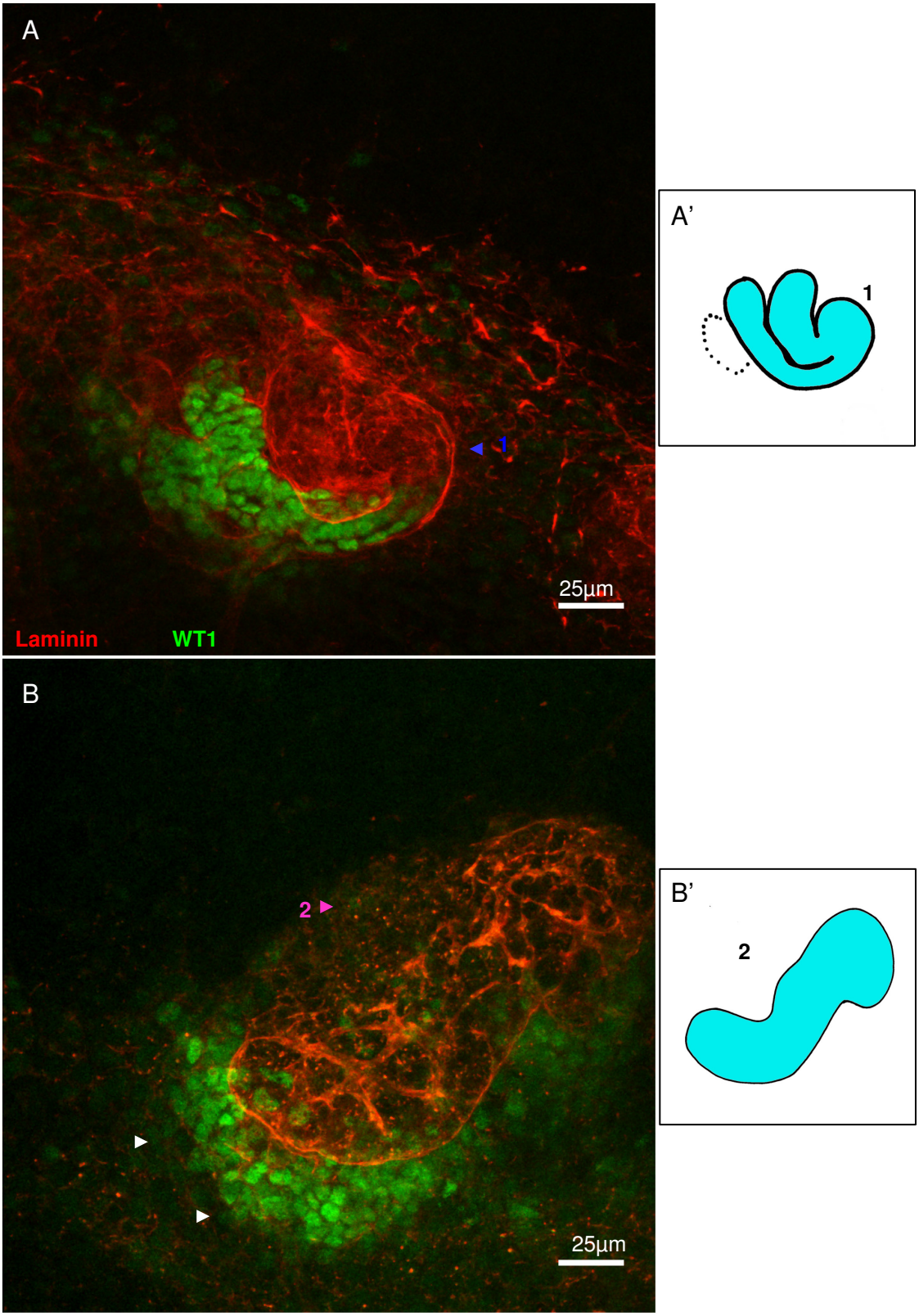
arrows and abnormal laminin deposition is indicated with turquoise arrows. (A') show a schematic representation of numbered nephrons in (A). The diagram was produced by careful inspection of nephrons at maximum resolution.

4.2.10 ROCK is necessary for normal positioning of the visceral and parietal epithelia.

The inversion of apicobasal polarity shown in the previous section is further illustrated by immunostaining for Wilms' tumour 1 homologue (WT1), a marker of developing podocytes. WT1-expressing, presumptive podocytes are normally located within the visceral epithelium and WT1 is also expressed at a lower level in the parietal epithelium. The process that structurally defines the parietal and visceral epithelia is the formation of the glomerular cleft. Glomerular cleft formation can be seen in (Fig.1.2), although these images show human samples. The glomerular cleft is lined with a basement membrane. In previous sections I have shown that glomerular cleft formation is interrupted as a result of blocking ROCK activity and the apicobasal polarity is disturbed in isolated nephrons. In this section I further investigate the formation of the glomerular cleft to determine whether the regulation of apicobasal polarity might play a role in this process.

Isolated metanephric mesenchyme induced by spinal cord form typical comma- and s-shaped bodies after 72hrs of culturing in control conditions (Fig.4.14A). The nephrons show high levels of WT1 expression in the visceral and parietal epithelia and the glomerular cleft also display clear deposition of laminin towards the cleft surface (Fig.4.14A). When ROCK is inhibited, nephrons form abnormally. WT1-expressing podocyte progenitors localised to the *outside* of the epithelial structures (Fig.4.14B,C) and there is no evidence of a flattened parietal epithelium or organised columnar epithelial cells resembling the presumptive podocytes in the visceral epithelium. Abnormal localisation of laminin to the interior of ROCK inhibitor nephrons is again evident. These data strongly support my hypothesis that the glomerular cleft is disturbed as a result of ROCK inhibition. In addition, as the apicobasal polarity of nephron cells was shown to be inverted in ROCK-inhibitor conditions, this suggests that the localisation of the WT1 expressing cells to the out side of the nephrogenic cysts could be a result of abnormal apicobasal polarity.

In whole kidney cultures, laminin localised in a more organotypical apicobasal manner in distal segments (Fig.4.5C,D). The morphologically abnormality shown by proximal segments, as has previously been described, makes the analysis of apicobasal polarity in these segments difficult.


A
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25μm
Laminin
WT1
A'
1
B
2
25μm
B'
2

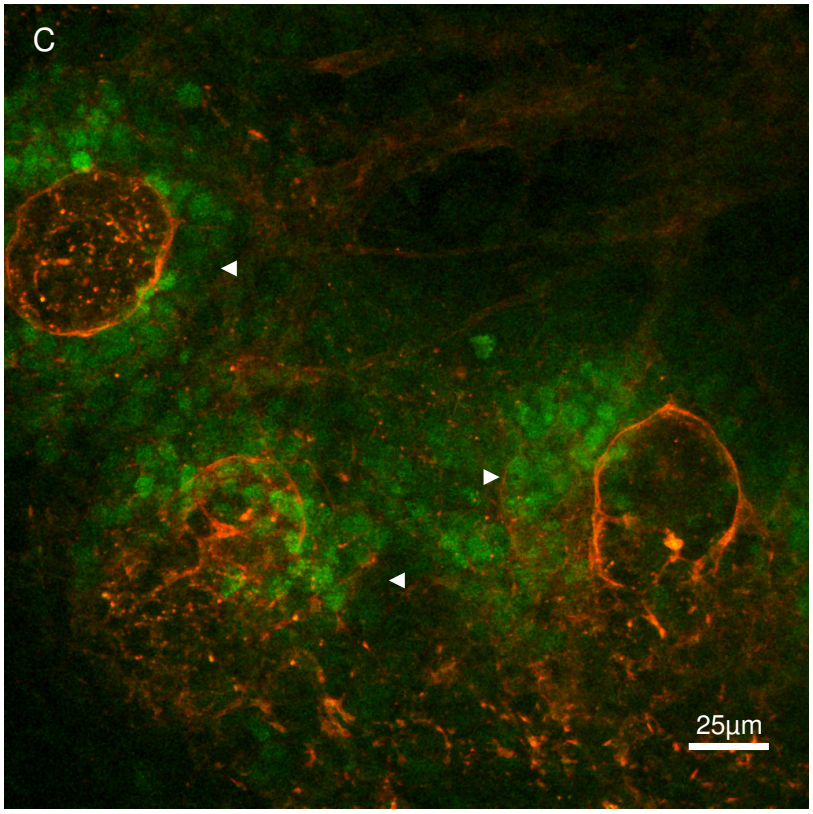

Fig.4.14 Inhibition of ROCK results in abnormal localisation of WT1 positive nuclei indicating abnormal apical-basal polarity

Fig.4.14 shows immunostaining showing the effects of ROCK inhibition on WT1 protein localisation. The morphology of nephrons formed in CKCM (A) was compared with that of nephrons formed in CKCM with 20µM Y-27632 (B and C). Nephrons were formed in spinal cord induced MM. All cultures were kept for 72hrs after which they were fixed and stained with rabbit anti-β-laminin, mouse anti-WT1, anti-rabbit-TRITC and anti-mouse-FITC. Blue arrowhead – normal nephron, magenta arrowhead – abnormal nephron, white arrowheads – abnormal WT1 positive nuclei distribution. (A' and B') show schematic representations of numbered nephrons in (A and B). Diagrams were produced by careful inspection of nephrons at maximum resolution.

4.2.11 ROCK is necessary for apicobasal polarity formation during development: a non-reversible process.

The effects of ROCK inhibition on apicobasal cell polarity raised the question whether ROCK is a necessary regulator of apicobasal cell polarity solely during nephron development or if inhibition of ROCK in more mature nephrons results in a transition from normal to inverted cell polarity. In MDCK cell experiments it has been shown that once MDCK cysts have polarised sufficiently after 4 days, it is no longer possible to inverse the polarity by blocking β1-integrin or expressing dominant negative Rac1 (Yu et al 2008). Here I investigate whether nephrons display a similar requirement for ROCK, where its function is only necessary during polarity development but not for subsequent polarity maintenance.

To investigate this possibility, spinal cord induced mesenchyme was cultured in control conditions for 72hrs to allow nephrons to mature to SB stage. At this point the ROCK inhibitor was administered for an additional 72hrs. Control cultures, which were cultured continuously in control medium, displayed normal apicobasal polarity as shown by basal expression of laminin and apical expression of CD15 (Fig.4.15A). Nephrons transferred to ROCK inhibiting conditions did not display inversion of polarity, although some form of epithelial degeneration was clearly visible throughout the samples (Fig.4.15B,C).
These results show that nephrons require ROCK for early apicobasal polarity development but the importance of ROCK for apicobasal polarity diminishes as the nephrons mature.

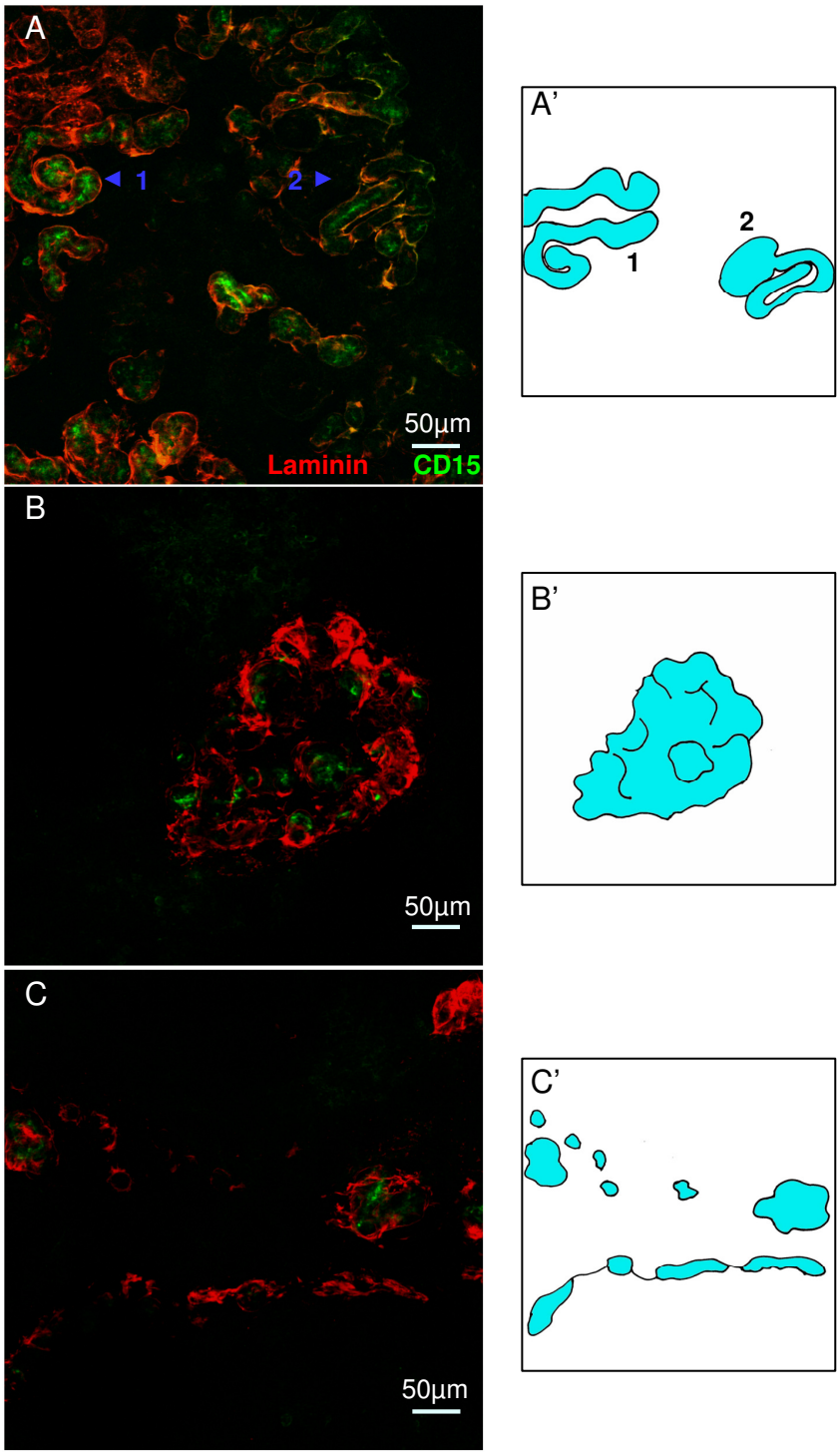

Fig.4.15 Inhibition of Rho-kinase does not reverse existing apicobasal polarity

Fig.4.15, (A) Normal nephrons are correctly polarised after 144hrs of culture as shown by tubular structures with correct basal and apical expression of laminin and CD15, blue arrowheads – nephrons 1-2. (B,C) Nephrons cultured for 72hrs in control conditions followed by 96hrs in ROCK inhibitor do not reverse polarity but do show severe loss of structure. Nephrons are illustrated using antibodies for laminin-TRITC and CD15-FITC. (A',B',C') show schematic representations of nephrons in (A,B,C). Diagrams were produced by careful inspection of nephrons at maximum resolution.

4.2.12 ROCK acts via actin-myosin contraction to regulate functions during nephron formation and maturation.

In the introduction to this chapter I briefly describe how ROCK regulates actin-myosin stress fibre formation via a pathway involving myosin light chain, myosin light chain phosphatase and myosin ATPase. In this section I tested whether the abnormalities seen in ROCK inhibitor nephrons can be mimicked by direct inhibition of actin-myosin contraction.

Using the same type of cultures (whole kidney cultures and spinal cord induced mesenchyme) I set up experiments testing whether the inhibition of myosin ATPase results in similar nephrogenic defects as the inhibition of ROCK. Myosin ATPase was inhibited using 2,3 butanedione monoxime (BDM) as previously optimised (Michael, Sweeney, and Davies 2005). Control cultures (Fig.4.16A) showed normal nephron formation whereas inhibition of myosin ATPase with BDM, resulted in severely inhibited nephron formation where no or very few nephrons formed (Fig.4.16B). In spinal cord induced mesenchyme nephrons formed normally in control conditions (Fig.4.16C) but in BDM conditions abnormal nephrons formed (Fig.4.16D). The effects of BDM appeared to be more severe than those of the ROCK inhibitor, although similarities could be seen between the effects if the two inhibitors. Inhibition of myosin ATPase did not produce the same morphological defects in the ureteric bud. The degree of abnormality displayed by the nephrogenic epithelia formed under BDM conditions made apicobasal polarity analyses difficult. The epithelia that formed showed no basic structures identifiable as nephrogenic and were as such not possible to classify into defined nephrons with either correct or abnormal apicobasally patterning.

Experiments were also carried out where BDM was added to whole kidney cultures, after 48hrs when renal vesicles (RV) had already formed. 56 nephrons from BDM conditions were examined and compared to previously described controls (Fig.4.12). The experimentally treated nephrons resembled the nephrons formed in ROCK inhibiting conditions in equivalent cultures (Fig.4.16E,F). 49% of BDM nephrons

showed structural abnormalities and 74% displayed aberrant E-cadherin expression (Fig.4.16G). The majority of BDM nephrons, 60%, were halted in the RV stage (Fig.4.16H). Half of BDM nephrons in RV stage displayed abnormalities, 47% (Fig.4.16I). The effects of BDM in kidney cultures appear to be more severe than the effects if ROCK inhibitors and it is more similar to what is seen when ROCK is inhibited in spinal cord-induced mesenchyme cultures. However, the apparent need for myosin ATPase before and after RV progression suggests similarities to the need for ROCK activity during nephrogenesis and for glomerular cleft formation.

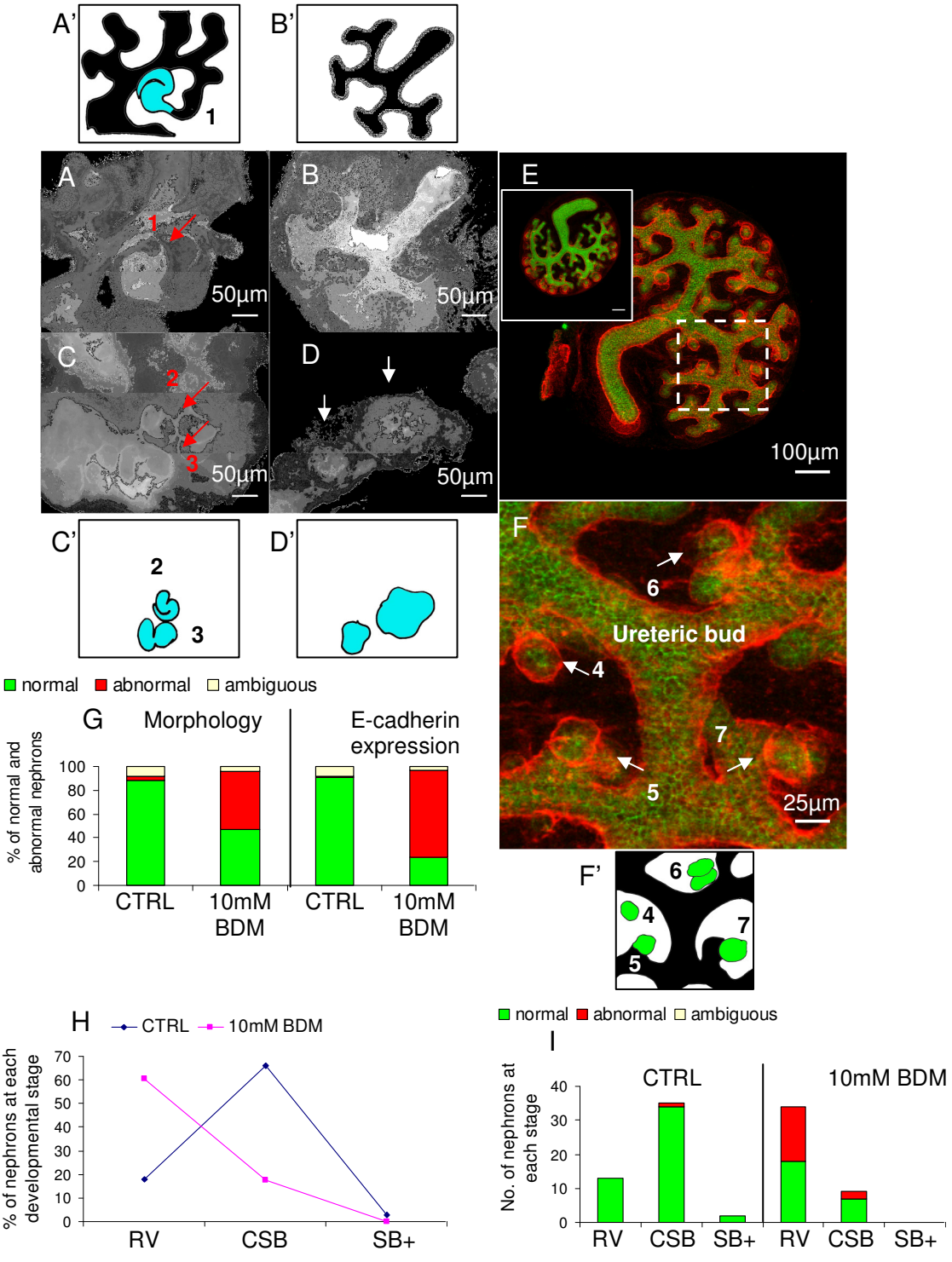

Fig.4.16 Direct inhibition of myosin activity mimics ROCK inhibitor effects.

Fig.4.16, (A) Kidney cultured in control conditions shows normal nephron formation, red arrow – nephron 1. (B) Kidney cultured in myosin ATPase inhibitor, BDM, has interrupted nephron formation. (C) Spinal cord induced mesenchyme in control conditions with normal nephrons, red arrows – nephrons 3-4. (D) In spinal cord induced mesenchyme with BDM, nephrons form abnormally with severe defects, white arrows. Cultures were maintained for 72hrs. (E,F) Confocal images of E-cadherin and laminin immunostaining, full size (E) and magnified (F) illustrate morphology and protein localisation in nephrons where BDM was added after 48hrs of culture. (E) inset image shows controls as shown in (Fig.6A). Controls for (E-I) are the same as for Fig.6. White arrows specify nephrons that display abnormalities in morphology and/or E-cadherin expression, nephrons 4-7. UB – ureteric bud. (G) 49% of nephrons formed in BDM had morphological abnormalities and 74% displayed abnormal E-cadherin expression. (H) Nephrons were also scored for their developmental stage as described in (Fig.6). Inhibition of BDM resulted in the majority of nephrons remaining at RV stage. (I) The majority of nephrons with abnormalities were found at an RV stage. (A'-D',F') show schematic representations of numbered nephrons in (A-D,F). Nephrons are in turquoise and the ureteric bud in black. Green segments of the nephron indicate parts positive for E-cadherin. Diagrams were produced by careful inspection of nephrons at maximum resolution. 58-CKCM nephrons and 56-BDM nephrons were analysed.

4.3 Chapter discussion

4.3.1 Inhibition of ROCK disturbs nephron and glomerular cleft formation: a polarity problem?

Nephrogenesis is a complex process that involves numerous different stages and mechanisms where it is likely that a wide array of regulatory pathways play important functions. In Chapter 4 I show that ROCK is needed during several of the stages of nephron development and I argue that ROCK is necessary for apicobasal polarity formation, glomerular cleft development and for the patterning of the nephrons' proximal-distal axis. I will discuss the roles of ROCK in the order that they seem to affect nephron development.

The formation of nephrons requires ROCK. At a moderate concentration of ROCK inhibitor, only 54% of the normal number of nephrons form; this is a significant reduction. When the ROCK inhibitor is added after the renal vesicles have already developed, 71% of the nephrons display some form of abnormality. If this reflects the degree of abnormality seen when ROCK inhibits nephrogenesis, this would mean that only 16% of the normal number of nephrons form properly. A decrease of this magnitude would severely reduce the functional capacity of the kidney.

One possibility is that a disruption of the mesenchymal-to-epithelial transition which gives rise to the nephrons could be the cause of the reduced nephrogenesis. An essential aspect of epitheliogenesis is the development of an apicobasal polarity (Davies and Garrod 1997). Kidney cells (MDCK cells) in 3D cultures have been shown to demand the Rho-GTPase family for normal apicobasal polarity formation (Rogers et al 2003; Yu et al 2008). It is clear that Rac1 is important for this polarity formation, possibly through activation via β1-integrin, but it is less clear what the role is of RhoA and its effector ROCK (Rogers et al 2003; Yu et al 2008). Rogers and colleagues clearly demonstrate that the expression of dominant negative RhoA, results in cellular cysts with inverted polarity (Rogers et al 2003). Yu and colleagues failed to replicate this finding using

RNAi to target *RhoA* or *ROCK-I* but they showed that a decrease in *RhoA* or ROCK could rescue the inverted polarity produced by the expression of dominant negative *Rac1* (Yu et al 2008). To understand the importance of RhoA and ROCK for polarity, this discrepancy must be resolved.

There is convincing evidence which argues that ROCK is needed for the apicobasal polarity formation in the mammary glands (Ewald et al 2008). The data shows that if ROCK is inhibited during mammary gland growth, the epithelial ducts fail to transit from a multilayered and growing state into stable monolayered and apicobasally polarized tubules (Ewald et al 2008). My data suggest that ROCK is important for apicobasal polarity formation during nephrogenesis. The inhibition of ROCK in spinal cord-induced mesenchyme leads to the development of nephrons with ectopic β-laminin deposition on the luminal side of the cysts. This lead to the hypothesis that the nephron, like the MDCK cells and the mammary glands also requires Rho or ROCK during the establishment of an apicobasal polarity. The development of an apicobasal polarity is a prerequisite to the formation of the renal vesicle. Therefore, without the apicobasal polarity it is unlikely that a glomerular cleft will form normally or that podocyte progenitor cells are localised normally. I show that this is the case. In whole kidney cultures, the main abnormality of the nephrons can be seen in the proximal regions where glomerular cleft formation is blocked. Nephrons with abnormal apicobasal polarity, in spinal cord induced mesenchyme, display cells with high levels of WT1 on the outside of the nephrogenic structures rather than the cells being located inside the structurally distinct visceral and parietal epithelia.

These data are difficult to explain mainly because the results that I have represents snapshots of nephrogenesis, rather than the continuous development of a nephron. Time-lapse microscopy could resolve this problem and the *WT1*$^{+/GFP}$ mouse would be a useful tool for this. I carried out initial experiments to show that the inhibition of ROCK also disturbed nephron development in this mouse strain. The purpose of this was to evaluate whether a real-time analysis could be carried out to demonstrate exactly what happens

during nephron formation and glomerular cleft formation. It was concluded that, although real-time microscopy would be possible on the *WT1*$^{+/GFP}$ mouse, there would definitely be a need for a marker of the basement membrane since light microscopy was insufficient to highlight the nephron structures. The combination of a basement membrane-fluorescent mouse with the *WT1*$^{+/GFP}$ would provide the optimal components to investigate the effects of ROCK on apicobasal polarity formation.

It is also important to discuss the differences between the results in spinal cord induced mesenchyme and whole kidney cultures. The induction of nephron formation by spinal cord is very efficient and in fact it appears to a more powerful inducer of nephron formation than the ureteric bud. This difference in efficiency might be explained by that the spinal cord continuously and widely induces the available mesenchyme whereas the ureteric bud induces nephron formation in a controlled manner where the optimum is not necessarily that all available mesenchyme is induced. The concentration of ROCK inhibitor that disturbed apicobasal polarity formation in spinal cord-induced mesenchyme almost completely blocked nephrogenesis in whole kidney cultures. I think that this difference is because the spinal cord seems to provide a stronger inductive signal and as such stimulates nephron formation to occur in spite of one of the mechanisms required (ROCK), not being present. The deposition of laminin in the mesenchyme of whole kidney cultures, where nephrogenesis would normally occur, supports the idea that nephron formation was attempted in these cultures but it failed as a result of ROCK inhibition. Further experiments highlighting the expression of nephron induction markers might add to this hypothesis.

Several molecular components have been identified that, if removed, results in blocked nephron development, e.g. *Wnt4*, α6β1 integrin and α-laminin (Klein et al 1988b; Sorokin et al 1990; Stark et al 1994). I propose that ROCK is also required. The mechanism of activation of ROCK is not known, nor is it known whether the requirement of ROCK is a property displayed by all epithelialising cells or whether ROCK is specifically activated in the nephrons. ROCK activation can be regulated via

Wnt-dependent planar cell polarity pathways (Habas, Dawid, and He 2003). As the Wnt/β-catenin pathway has been demonstrated to induce nephron formation, it would be interesting to further investigate whether a planar cell polarity pathway is also important. Osafune and colleagues has published some data indicating that a planar cell polarity pathway is important for the size of nephron progenitor cell cultures in vitro, however, more work is required to cement this hypothesis (Osafune et al 2006). Park and colleagues showed that the Wnt/β-catenin pathway is sufficient to induce nephron formation but the epithelial-to-mesenchymal transition does not take place (Park, Valerius, and McMahon 2007). Perhaps this indicates that a non-canonical pathway is also necessary where for example, ROCK might play a role during proper apicobasal polarity formation.

One possible way to determine whether ROCK is activated by the Wnt/non-canonical planar cell polarity pathway would be by blocking a crucial component of the pathway and measuring the level of ROCK activation. Dishevelled would provide a good candidate gene to block but as it is involved in the Wnt/β-catenin dependent pathway, this would have to be rescued in order to induce nephron formation. GSK3β inhibitors have been optimised and shown to work in kidney cultures; the use of such inhibitors could rescue the β-catenin pathway (Davies and Garrod 1995; Kuure et al 2007). This experiment could show whether blocking the planar cell polarity affects the activation of ROCK in nephrons or the kidney.

4.3.2 ROCK is necessary for proximal-distal axis formation.

The development of the proximal-distal axis in the nephron requires ROCK. Inhibition of ROCK before or after the renal vesicle stage produces nephrons with abnormally patterned proximal-distal axes. Proteins that are normally found in a segment-specific pattern were detected in the whole of the tubular segment of the nephron instead of being restricted to their appropriate domain. This abnormal patterning of markers even

included WT1 which is normally only expressed in the parietal and visceral epithelia. In the results section I provided two alternative explanations for this. Firstly, there is the possibility that the cells actually differentiate abnormally and therefore they express markers for both distal and proximal segments. The second hypothesis suggested that it could be a failure of segregation of proximal and distal cells, and not differentiation, which resulted in the mixing of segment markers. I also presented the possibility that a failure to segregate properly might lead to the first hypothesis where the non-segregated cells start to acquire an abnormal identity due to their environment and therefore express ectopic genes. My interpretation is that the co-localisation of WT1 and CD15 in tubular segments of the nephron, as well as the presence of CD15 in E-cadherin positive cells, suggests that abnormal differentiation is taking place. The mechanism that is disturbed to produce these results is still to be determined. To speculate on this point, it is a strong possibility that the abnormal differentiation is a result of abnormal segregation of cells. I find it plausible that the abnormal positioning of a cell might result in the cell being in an environment that is inductive towards the differentiation down a segment-specific pathway. Alternatively, it is also worthwhile considering that it is not the environment that stimulates differentiation, but instead it might be that all cells in the renal vesicle attempt to differentiate down all pathways and the segregation of cells forces them into environments that are inhibitory towards all fates other than that of the segment which they are in.

It is possible that ROCK is important for cell segregation. Sahai and Marshall showed that ROCK can disrupt adherens junctions (Sahai and Marshall 2002). A number of cadherins show segment specific expression along the proximal-distal axis of the nephron although disruption of cadherin function has had quite limited phenotypes (Cho et al 1998; Mah et al 2000; Dahl et al 2002). Differential cadherin expression has, in other systems, been shown to result in cell sorting as explained by the differential adhesion hypothesis where cells expressing different cadherins or different levels of the same cadherin can successfully segregate (Foty and Steinberg 2005; Steinberg 2007). Interestingly, cadherin mediated segregation of cell populations might not necessarily be

a result solely based on differential cadherin expression but differences in the number of cadherin molecules may play a significant role, as discussed by (Foty and Steinberg 2005). The possibility of non-sorting of cells is attractive as it could be accountable for the lack of nephron segmentation and cells expressing markers for more than one nephron segment.

These data are not the first pieces of evidence showing the plasticity of nephron cells. Reggiani and colleagues showed that *Irx3* is necessary for the differentiation of a particular segment in the Xenopus pronephros (Reggiani et al 2007). Deletion of *Irx3* lead to the loss of a specific segment differentiating as shown by the loss of expression a segment-specific marker (Reggiani et al 2007). In addition, supplying *Irx3* mRNA resulted in the ectopic differentiation of the *Irx3* regulated segment (Reggiani et al 2007). It is uncertain when (or if) cells acquire a non-plastic segment-specific identity, although it is known that the *Six2* positive nephron progenitor cells can contribute to all segments of the nephron (Kobayashi 2008).

Regardless of which mechanism it is that disturbs the nephron proximal-distal axis patterning it is interesting to note that many segment-specific genes are still activated. I showed that L1-cell adhesion molecule, WT1, CD15, E-cadherin (immunofluorescence protein studies), *Sglt2*, *Hey1*, *Cadherin-6*, *Irx3*, *Brn1*, *THP*, and *Hunk* (RT-PCR/PCR studies) were all present/expressed in kidneys where ROCK had been inhibited. In the early nephron these proteins/genes represent every segment that is present at this stage. I speculate that, together with the data showing abnormal segment patterning with ectopic gene expression, these data indicate that ROCK is not likely to directly regulate cellular differentiation in the nephron. This would fit with the hypothesis that all the cells in the nephron have the capability to produce any portion of the nephron (Kobayashi 2008). I propose that ROCK is a necessary component in a mechanism that allows the cells in the nephron to pattern themselves according to their differentiation so that a segmented proximal-distal axis is produced.

If abnormal differentiation is a result of the failure of cells to segregate normally it could be experimentally tested. One way to test this hypothesis would be to produce a conditional transgenic model. The idea would be to drive a rather complex construct that excises a floxed endogenous *cadherin-6*, and activates a transgenically inserted *GFP-tagged E-cadherin* driven under the endogenous *cadherin-6* promoter. *Cadherin-6* is normally only expressed in the proximal regions and *E-cadherin* only in the distal segments. This model would produce cells that are entirely proximal, apart from that they express *E-cadherin* instead of *cadherin-6*. This would demonstrate whether the proximal E-cadherin positive cells would adhere to distal E-cadherin positive cells and express genes for both proximal and distal lineages.

The question remains whether the requirement for ROCK during nephrogenesis is a result of ROCK being necessary for a planar cell polarity pathway.

4.3.3 ROCK through myosin and actin-myosin contraction.

The demonstration that ROCK regulates nephrogenesis and maturation prompts the question about what mechanism it is that ROCK uses during these processes. I showed that the direct inhibition of myosin-ATPase, which is required for actin-myosin contraction, results in a strong blockage of nephron formation in whole kidney cultures, reminiscent of high concentrations of ROCK inhibitor. The ureteric bud was not affected in the same way as when ROCK is inhibited. The ureteric bud was not bloated, in fact it appeared to be smaller as if it had halted its development, as previously shown by Michael, Sweeney and Davies (Michael, Sweeney, and Davies 2005). Epitheliogenesis could be stimulated in spinal cord induced mesenchyme, again suggesting that nephron formation is more strongly induced in this system. The epithelial structures that did form in these cultures were structurally deformed, making it impossible to determine whether apicobasal polarity formation was affected. In experiments where the myosin-ATPase was added after 48hrs, when renal vesicles had formed, the nephrons displayed

structural malformations and the majority of nephrons displayed abnormal expression of E-cadherin. Similarly to the ROCK treated nephrons, the main portion of nephrons that were abnormal were in renal vesicle stage. I suggest that the effects of inhibiting myosin-ATPase in the kidney might be much more severe than inhibiting ROCK. Inhibition of ROCK will not completely stop actin-myosin contraction since myosin light chain can still be activated other mechanisms, for example myosin light chain kinase, discussed by Amano and colleagues (Amano et al 1996), see Diagram4.1. This means that inhibition of ROCK would only block ROCK regulation of actin-myosin contraction whereas other pathways might remain unaffected. This could account for the differences in the severity of effects between the two inhibitors.

Chapter 5

Discussion

5.0 Discussion

The kidney develops via several intricate stages where the ureteric bud continuously branches and elongates as a result of positive and inhibitory signals from the metanephric mesenchyme, and as it does this it also induces nephrons to form in the virgin mesenchyme that it encounters. The mechanisms that contribute towards the development of the kidney very much represent a grey area of understanding and they require significantly more research. Rac1-GTPase (Rac1) and Rho-kinase (ROCK) were chosen as highly relevant proteins to investigate based on previously published experiments (from the Davies lab and from other research groups), and because they carry out mechanically interesting functions.

Using kidney organ culture techniques and specific inhibitors, I targeted Rac1 and ROCK in vitro in order to determine what functions these proteins play during specific stages of kidney development. I have generated data which suggest that normal Rac1-activity is necessary for normal proliferation rates in the kidney; a process I showed to be essential for normal branching morphogenesis. I showed that ROCK is necessary for several different stages of nephron development and the development of two sets of nephron polarities; the apicobasal and the proximal-distal. In fact, inhibition of ROCK blocks nephrogenesis, glomerular cleft formation and proximal-distal nephron axis development. These data are separately discussed below in light of published data and with suggestions for improvements and further experiments.

Using in vitro cultures to model in vivo morphogenesis or the development of an organ requires appropriate consideration regarding the validity of such a model and the extrapolations and interpretations that can be made towards the in vivo situation. Here I have used two different systems; the spinal cord-induced mesenchyme to study nephron development and the whole-kidney culture system for a general approach to view the kidney as a whole. It is also possible to culture ureteric buds, isolated from metanephric

mesenchyme, and induce them to branch within three-dimensional gels consisting of collagen and basement membrane components (Qiao, Sakurai and Nigam 1999). Manipulations of a tissue for experimental purposes inherently results in the separation from the in vivo situation, yet these culturing techniques can provide valuable insights into the signalling within and between tissues. Arguably, the culturing of whole kidney-rudiments provides a good model for in vivo kidney development as ureteric bud branching and nephron formation resembles that which occurs in vivo, in particularly when considering the early stages of kidney development (Saxen 1987). However, several aspects makes whole-kidney in vitro cultures a poor model for nephron development and ureteric bud branching and these are crucial to bear in mind when discussing the findings of this thesis. Firstly, the isolation of the kidney rudiments from the surrounding embryonic tissues results in the immediate disruption of tissue-to-tissue signalling and significantly the interruption of proper vascularisation. The disturbed vascularisation will have major impacts on the supply of growth factors and nutrients to the growing cells. Additionally, a crucial step of nephron formation is the development of the glomerulus, a structure contained within the renal corpuscle. The glomerulus is required for the supply of blood to the nephron, thus enabling blood filtration. The failure of vascularisation and the failure to form the glomerulus is a limiting factor for using in vitro kidney cultures as a method to understand in vivo development.

This divergence between in vitro and in vivo development has recently become an increasingly interesting topic. The lack of a blood supply will result in a loss of urine production and normal fluid flow within the nephron and the ureteric bud. This might have impacts on the regulation of developmental signalling programs. As discussed in-depth in the introduction, Wnt signalling is an important component of kidney development (Stark et al 1994; Park Valerius and McMahon 2007). It has been demonstrated that Wnt signalling can be controlled by cilia where the motion of cilia, stimulated by for example the flow of a fluid, regulates the activation of Wnt signalling, as reviewed in (Germino 2005). Epithelial tubes such as the tubules of the nephron and the collecting duct system are lined with cilia that through fluid flow could thus regulate

Wnt signalling, discussed in (Germino 2005). As previously mentioned, components of Wnt signalling pathways as well as of the Fat-protocadherin pathway have been found within both the ureteric bud and the nephrons (Marose et al 2008; Saburi et al 2008). Recently, it has been demonstrated that Wnt7b is capable of regulating the mitotic angles of cells in both nephron and the ureteric bud epithelia (Yu et al 2009), something that has also been shown to be coordinated by the Fat-protocadherin pathway (Saburi et al 2008). The possibility that the flow of fluids, through developing epithelial tubules, could regulate Wnt signalling in the kidney is an important point to consider not only from a general developmental biology perspective but especially when using in vitro culture systems to study kidney development.

This potential difference between in vivo and in vitro kidney development is important to remember for the discussion of this thesis, however, as the stages of kidney development discussed herein are of an immature stage, it is arguable that potential differences formed by fluid flow would be minimal.

5.1 Rac1 is necessary for proliferation: and something else?

Normal Rac1 activity is necessary for kidney proliferation, as shown in Chapter 3. Inhibition of Rac1, using moderate concentrations of Rac1 inhibitor resulted in a 24-34% reduction of cells progressing through DNA synthesis. This was not a surprising feature of Rac1 inhibition as Rac1 has been shown to be necessary for proliferation in other systems, such as in fibroblast, endothelial, and haematopoietic stem cells (Moore et al 1997; Mettouchi et al 2001; Gu et al 2003).

Interestingly, I showed that the inhibition of Rac1 resulted in morphological defects in the kidney, mainly evident by the reduced ureteric bud branching and decreased overall growth. Michael and Davies showed that proliferation is a requirement for ureteric bud induction and that proliferating cells are more frequently detected in the ureteric bud tips compared to the ureteric bud stalk regions; the latter was sensitive to the activity of the known ramogen GDNF (Michael and Davies 2004). Regionalised high levels of

proliferation has also been shown in the mammary glands at the ends of ducts, and both proliferation and Rac1 were separately shown to be required for duct formation (Ewald et al 2008). In a different system, where nephron progenitor cells are cultured in a two dimensional system and induced by Wnt4, Rac1 has been shown to be important and the inhibition of Rac1 activity resulted in reduced culture sizes (Osafune et al 2006). Neither Osafune and colleagues, nor Ewald and colleagues attempted to determine by what mechanism it was that Rac1 functioned, nor did they connect Rac1 to the regulation of proliferation (Osafune et al 2006; Ewald et al 2008). I showed that the inhibition of Rac1 in whole kidney cultures resulted in smaller kidneys with fewer branches and that these cultures had reduced numbers of BrdU positive cells.

The effects of Rac1 inhibition on kidney development were investigated further in order to determine whether the reduced branching and growth was a result of the decreased proliferation. If the reduced branching produced by inhibiting Rac1 were to be caused solely by tissue-wide decreased proliferation, then it should be possible to mimic this by directly decreasing proliferation. If it could not be mimicked that would mean that Rac1 might play additional roles during kidney development. I found that direct inhibition of proliferation resulted in decreased branching and growth. Interestingly, when the proliferation was reduced by a similar proportion to that caused by Rac1 inhibition, the decrease in branching was greater by direct inhibition of proliferation. If the data are interpreted in a straightforward manner, it would mean that Rac1 activity must normally negatively affect ureteric bud branching as well as regulating proliferation. The inhibition of Rac1 would result in a dual effect since the branching is released from the Rac1-controlled negative regulation as well as being subjected to decreasing proliferation rates.

I think that the next logical experiment would be to produce a model where Rac1 activity can be specifically regulated in the ureteric bud tips. The ureteric bud branches at the tips 94% of the time (Watanabe and Costantini 2004) and the tips display significantly higher levels of proliferation compared to stalk regions (Michael and

Davies 2004). This suggests that, if Rac1 is necessary for regulating proliferation and if this is a mechanism for controlling branching, then it would be expected that a specific reduction of Rac1 activity in the ureteric bud tips would decrease branching. It would be essential to confirm this by, also in a tip-specific manner, blocking cell cycling via a different mechanism. This could be achieved by expressing a cell cycling inhibiting protein (e.g. p27Kip1), under the control of a tip-specific gene (e.g. Wnt11) (Kispert et al 1996; Goukassian et al 2001). It is still possible that Rac1 performs other functions in the ureteric bud tips and we will therefore generate the same data as from the organ-wide inhibition of Rac1 and proliferation. To determine whether this is the case, it would be necessary to start elucidating what downstream regulators it is that Rac1 activates in the kidney and from there investigate how these, individually, contribute to kidney development. It is also possible that the inhibition of Rac1 does not reflect a true direct pathway between Rac1 and proliferation; it might equally well reveal a general necessity for Rac1 activity for cell viability. The same argument is applicable for the effects of Rac1 inhibition on ureteric bud branching. I find these explanations unlikely since relatively small differences in Rac1 inhibitor concentrations could affect branching to a significantly different extents.

Apart from the branching, proliferation and overall growth, there were no other detected effects from inhibiting Rac1. Osafune and colleagues showed that inhibition of JNK1 or JNK2 lead to a failure of the nephron progenitor cells to differentiate and express genes that indicate nephron maturation (Osafune et al 2006). Rac1 can act upstream of JNK in the planar cell polarity pathway (Habas, Dawid, and He 2003). I generated no data that suggested that Rac1 is a regulator of nephron differentiation. On the contrary I found that the nephrons appeared morphologically normal and displayed markers of typical differentiation.

Osafune and colleagues proposed that a planar cell polarity pathway regulates not only the size of their nephron progenitor cell cultures, but also the differentiation of these

cells towards different nephron lineages (Osafune et al 2006). I showed that Rac1 is unlikely to regulate nephron formation or subsequent differentiation.
It cannot be ruled out that Rac1 is nevertheless a necessary component of nephron differentiation and that a general abundance of active Rac1 is present and that only very low levels of Rac1 are required and that I do not achieve such complete inhibition by using the Rac1 inhibitor.

To further explore the possibility that Rac1 is regulated by a Wnt/planar cell polarity pathway I would devise a similar experiment as that explained in Chapter 4. There I proposed the disruption of Dishevelled, and the simultaneous rescue of β-catenin signalling as an approach to determine whether ROCK is downstream of a Wnt/planar cell polarity pathway although in this case, Rac1 activity would be assayed instead of ROCK.

One mechanism of morphogenesis so far not mentioned, which is highly relevant when considering Rac1 and proliferation, is that of programmed cell death. A substantial number of cells, up to 3%, display apoptotic features and as the cells degrade at a relatively rapid rate, 1-2hrs, this could indicate that perhaps as many as 50% of cells that form in the kidney die by apoptosis (Coles, Burn and Raff 1993). The majority of apoptotic cells are detected in the metanephric mesenchyme and in particular those regions that surround the nephrons, although strikingly, apoptotic cells were also found within nephrons (Coles, Burn and Raff 1993). The importance of cell death for kidney morphogenesis was convincingly demonstrated through the inhibition of apoptosis in the kidney (Araki et al 1999). Inhibition of caspase activity resulted in abnormal branching morphogenesis of the ureteric bud as well as reduced nephrogenesis (Araki et al 1999). A particularly interesting aspect that supports the possibility that apoptosis is important for kidney morphogenesis was the detection of apoptotic cells in the lower portion of the s-shaped body; that portion of the nephron which eventually gives rise to the proximal parts of the nephron (Coles, Burn and Raff 1993). Apoptotic nuclei were frequently detected in this nephron segment (Cole, Burn and Raff 1993). This finding is of interest

because there is a lack of models explaining the mechanisms of morphogenesis that regulate the formation of the s-shaped body including the development of the glomerular cleft and the flattened parietal epithelium. As discussed in the introduction, the mechanisms involved in the morphogenesis of the nephron are largely unknown. Specific locations of apoptosis could clearly play an important part in shaping the structure of the nephron epithelium and should as such be considered as a possible mechanism of nephron morphogenesis.

In connection with Rac1, if cell death is a mechanism of nephron morphogenesis, then one could argue that cellular proliferation would likely be under a similar level of control in order to produce morphologically normal nephrons and ureteric buds. It would be of interest to study the precise location and orientation of dividing cells as well as apoptotic cells within the renal vesicle, through to the s-shaped body, in order to determine whether a replicable pattern is detected.

5.2 Rho-kinase and nephrogenesis

In this thesis I demonstrated that Rho-kinase (ROCK) was important for renal vesicle formation, glomerular cleft development and for the patterning of cells along the proximal-distal axis of the nephron. Previous findings have shown that ROCK plays an important part during kidney development (Michael and Davies 2004; Meyer et al 2006). Inhibition of Rho-kinase in kidney cultures resulted in kidneys that had visibly bloated ureteric buds (Michael, Sweeney, and Davies 2005) and that had actually also increased in overall size, probably as a result of higher proliferation rates (Meyer et al 2006). Planar cell polarity pathways acting via a RhoA-dependent planar cell polarity pathway (Osafune et al 2006) and a Wnt-independent, protocadherin-dependent planar cell polarity pathway (Saburi et al 2008) have been suggested to be acting in the kidney. The findings of Osafune and colleagues show that if active *RhoA* or ROCK were reduced/inhibited in nephron progenitor cell cultures, this led to larger cultures

compared to controls (Osafune et al 2006). The mechanism for this was not investigated but it is possible that increased proliferation, as seen in whole kidneys when ROCK was inhibited (Meyer et al 2006), could at least in part account for the size difference. Inhibition of ROCK did not affect the differentiation of these cells towards expressing nephron segment markers (Osafune et al 2006).

I have shown that ROCK was important during renal vesicle formation. Inhibition of ROCK at high inhibitor concentrations almost completely blocked renal vesicle formation, and at lower concentrations there was still a large decrease. These effects were shown to be independent of the ureteric bud deformities caused by the inhibitor. A possibility is that ROCK was required during apicobasal polarity formation, and if that was the case, this could explain why very few renal vesicles were formed and why laminin was being deposited on the luminal epithelial surfaces when ROCK was inhibited. Interestingly, the inhibition of ROCK did not disturb the apicobasal polarity in nephrons with a properly established polarity, suggesting that the necessity for ROCK reflects a developmental and not a maintenance process. This correlates well with Yu's suggestion that the knockdown of ROCK could only rescue inverted MDCK cell cysts in the first few days and vice versa; inverted polarity could only be induced before the cysts developed a stable apicobasal polarity (Yu et al 2008). In the introduction to Chapter 4 I described other systems where ROCK has been implicated to regulate apicobasal polarity. Perhaps most notably of the examples, is the requirement of ROCK to stabilise the apicobasal polarity of mammary gland ducts (Ewald et al 2008). It is important to point out that this process differs from the mesenchymal-to-epithelial transition that produces the nephrons. The nephrons form via bona fide epitheliogenesis whereas the mammary gland ducts develop by a process that more closely resembles the sprouting of cells from an already existing epithelium as seen during MDCK cell tubulogenesis (Pollack, Runyan, and Mostov 1998; Ewald et al 2008). It is difficult to determine at what level parallels can be drawn between these two systems.

Ideally, if nephron progenitor cells could be harvested and induced to form nephrons in a three dimensional matrix, without the presence of a large number of other cells, this

could provide the basis of a high-resolution study, similar to those carried out on MDCK cells, where it would be possible to properly define the need of ROCK during apicobasal polarity formation. There is a range of experiments that could further elucidate the role of ROCK during renal vesicle formation. Firstly, a new system has been developed where kidney cells are dissociated to a single cell suspension and then reaggregated to form ureteric bud structures and mesenchyme capable of normal growth and induction (Unbekandt and Davies, unpublished). The strength of this system comes from that the single-cell suspension stage is highly accessible for transfection with either siRNAs or whole plasmids. The kidney has otherwise proven to be highly resistant to in vitro transfection. This system would immediately open up the possibility to specifically knock down ROCK-I and ROCK-II separately, as well as to target a large array of different ROCK effectors. It would also allow for the transfection of constructs carrying inducible constitutively active forms of ROCK or ROCK effectors, immune to the effects of the ROCK inhibitor, thus providing a possible mechanism to rescue nephrons from ROCK inhibition.

One of the interesting aspects of this thesis is that ROCK is needed for several different processes during nephrogenesis. The second mechanism that was disrupted in ROCK inhibitor nephrons was the formation of the glomerular cleft. Initially, I considered the possibility that this effect could be secondary to the abnormal renal vesicle formation. However, adding the ROCK inhibitor at a stage where renal vesicles had already developed, lead to the same abnormalities being detected. This indicated that the abnormal glomerular clefts formed by ROCK inhibition were not a result of disturbed renal vesicle development. Instead, this could reflect another ROCK-dependent process. It could of course still be the same mechanism that was regulated by ROCK and that this mechanism was used for different purposes at separate time points. The abnormal positioning of the WT1-positive cells in nephrons forming in ROCK inhibitor conditions suggested that there was a failure of the development of the presumptive renal corpuscle. Since the WT1-positive cells were located on the outside of the nephron, this might indicate that another polarity-based process was interrupted by the ROCK inhibition.

Nephrons without a renal corpuscle would most probably interact poorly, if at all, with the blood vessels.

The third stage of nephron development that is affected by the inhibition of ROCK is during the development of the proximal-distal axis. Knockouts of *Notch2* and *Irx3* have shown that single genes can affect a whole nephron segment and either result in a loss of the whole segment structure or an expansion of neighbouring segment identities (Cheng et al 2007; Reggiani et al 2007). In fact, it is now known that a population of *Six2*-expressing cells are capable of producing all the segments of the nephron (Kobayashi 2008), something that was also suggested by Osafune et al (2006) using their in vitro cell culture system of *Salll1*-positive cells. These findings really demonstrated the plasticity of nephron progenitor cell populations. I show that by inhibiting ROCK, nephrons failed to properly define their proximal-distal axis. The abnormally patterned nephrons were not simply showing cells of different segment types that were intimately mingling. Cells were shown to ectopically express markers for more than one nephron structure. This suggested that cells were differentiating abnormally as a result of ROCK inhibition. However, by monitoring the gene expression of several nephron segment-specific genes, in kidneys treated with ROCK inhibitor, I demonstrated that the inhibition of ROCK did not result in the loss of expression of these genes. This indicated that, although ROCK inhibition affected the final patterning of differentiation, it might not be a significant regulator of the induction of gene expression. This prompted the hypothesis that cells in a nephron might require a mechanism whereby they are appropriately patterned along the proximal-distal axis and that this patterning is also important for the cells' normal differentiation. I suggest that the inhibition of ROCK disrupts the physical patterning of cells and this leads to cells being located in the wrong place and acquiring ectopic gene expression.

What mechanism is resulting in the abnormal segregation of cells along the proximal-distal axis? One possibility was presented in Chapter 4 where I suggested that cell sorting, by differential adhesion or another mechanism, might be blocked by ROCK

inhibition. ROCK has been shown to be able to negatively affect adherens junctions (Sahai and Marshall 2002), see Diagram4.2 in Chapter 4. Here I inhibited ROCK and as a result, possibly increasing adherens junctions stability, thus restricting cell-sorting. It is unlikely that the proximal-distal patterning functions via only this system but it is an important line of investigation. The abnormally patterned nephrons also displayed several morphological defects. A portion of nephrons fused to the ureteric bud at more than one site. This would suggest that the ability to fuse, which is normally occurring in the distal portion of the nephron, was expanded to other regions of the nephron. Whether this was a consequence of the abnormal patterning, remains to be shown. One interesting effect is of course the expansion of E-cadherin from the distal domains to proximal domains. It has not been formally demonstrated whether E-cadherin plays a role during nephron-ureteric bud fusion. It is interesting that the expression of E-cadherin is initially restricted to the region of the nephron that will fuse to the, also E-cadherin positive, ureteric bud. One possibility is that Wnt signalling is affecting the patterning of cadherins in the nephrons since, in MDCK cells, Wnt-signalling results in lower levels of cadherin-6 (Stewart, Barth, and Nelson 2000). This might have a mechanical importance since cadherin-6 is normally detected in the proximal regions of the renal vesicle and not in the distal regions that are near to the Wnt expressing ureteric bud (Cho et al 1998). The experiment stipulated to investigate whether cell-adhesion is important for proximal-distal nephron patterning (see Chapter 4 Discussion), might also answer what role E-cadherin has for nephron fusion to the ureteric bud since E-cadherin would be ectopically expressed in proximal nephron segments.

Another morphological defect in the ROCK inhibitor cultures was the development of cyst-like nephrons. Cystogenesis in nephrons has previously been demonstrated to be the result of disrupted planar cell polarity. The proto-cadherin dependent planar cell polarity pathway has been shown to be involved in the regulation of the mitotic axis in nephrons (Saburi et al 2008). Knockouts of *Fat4*, an integral component of this pathway, resulted in cystic nephrons that resembled those found in polycystic kidneys (Saburi et al 2008). The regulation of the mitotic axis has actually been directly linked to polycystic kidney

disease (Fischer et al 2006). Polycystic kidney disease has also been linked to microtubule stability (Woo et al 1994). Since ROCK is a regulator of both the cytoskeleton and the proliferation rates in the kidney, these could be important directions to investigate to better understand polycystic kidney disease. I suggest that it would be important to demonstrate whether the inhibition of ROCK also affects mitotic axis.

The finding that ROCK is necessary for nephron development spurs questions about what mechanism it is that ROCK uses for the different processes. To answer this question, I attempted to block the activity of one of ROCK's downstream effectors. Diagram4.1 in Chapter 4 shows some of the important components that ROCK uses to regulate the formation and contraction of actin-myosin. I blocked myosin ATPase and found that this produced nephrons that shared similarities with the ones generated in ROCK inhibitor conditions. Inhibition of myosin ATPase disrupted renal vesicle formation. This suggests that ROCK might be important for renal vesicle formation through a pathway that regulates the formation and contraction of stress fibres. Secondly, when myosin ATPase was blocked after renal vesicle formation, the nephrons that developed also showed abnormal patterning along the proximal-distal axis. This indicated that the ROCK-dependent mechanism, that is important for proximal-distal patterning, might be dependent on actin-myosin contraction. If this is the case, this would show that some form of mechanical force is needed for normal patterning. As mentioned, the there were also differences in the effects produced by inhibiting ROCK and myosin ATPase. Most notable of the differences was the almost complete block of nephron maturation past renal vesicle stage. I propose that the differences between inhibiting ROCK and myosin ATPase was that, when ROCK was inactivated, other pathways were still capable of stimulating actin-myosin contraction (see Diagram4.1 in Chapter 4. When myosin ATPase was inhibited, actin-myosin contraction would have been more directly affected. If actin-myosin contraction and stress fibre formation is important for nephron development, these processes would be much more severely inhibited by the direct targeting of myosin ATPase compared to targeting ROCK. It is also possible that the differences between inhibiting myosin ATPase and ROCK could

result from that ROCK can also affect other pathways that could be important for nephrogenesis. This requires further research to determine if this is the case.

There is another possible mechanism that is only briefly mentioned in this thesis. Although the Wnt/β-catenin signalling pathway has been shown to be essential for nephron induction, little has been discussed regarding the pool of β-catenin which is located in the adherens junctions. It should not be ruled out that ROCK might indirectly affect β-catenin signalling via its control on adherens junctions. The expansion of E-cadherin expression could lead to effects on β-catenin signalling as a large proportion of β-catenin could be directed towards the adherens junctions. It is arguable to what extent this might affect β-catenin signalling as a whole, but it would be unwise to rule out this possibility until proven otherwise.

5.3 *Finale*

There is a need to better understand the relationship between the genetic and the morphogenetic processes that operate during kidney development. Elucidating the roles of Rac1 and ROCK represents the early steps that provide a bridge between the molecular elements and the mechanical forces that are necessary in order to regulate, for example, the branching of the ureteric bud or the development of the nephron. In this thesis I presented Rac1 and ROCK as important proteins for several of the events that take place in the embryonic kidney. Proliferation and the contraction of stress-fibres were suggested as their respective mechanism, which they act through to contribute to the ureteric bud branching and growth, and for several stages of nephrogenesis. The next step is to connect Rac1 and ROCK, proteins that are quite far down the ladder, with those that are at the top. How is the activity of Rac1 or ROCK turned on or turned off? Is there a connection with genetic pathways that have already been identified as being important? By answering these questions we will achieve a much improved perspective

on how some fundamental processes are regulated during kidney development, and perhaps also how other organs and tissues develop.

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Appendix 1

Developmental plasticity and regenerative capacity in the renal ureteric bud/collecting duct system

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Developmental plasticity and regenerative capacity in the renal ureteric bud/collecting duct system

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Branching morphogenesis of epithelia is an important mechanism in animal development, being responsible for the characteristic architectures of glandular organs such as kidney, lung, prostate and salivary gland. In these systems, new branches usually arise at the tips of existing branches. Recent studies, particularly in kidney, have shown that tip cells express a set of genes distinct from those in the stalks. Tip cells also undergo most cell proliferation, daughter cells either remaining in the tip or being left behind as the tips advance, to differentiate and contribute to new stalk. Published time-lapse observations have suggested, though, that new branches may be able to arise from stalks. This happens so rarely, however, that it is not clear whether this reflects true plasticity and reversal of differentiation, or whether it is just an occasional instance of groups of tip cells being 'left behind' by error in a mainly stalk zone. To determine whether cells that have differentiated into stalks really do retain the ability to make new tips, we have removed existing tips from stalks, verified that the stalks are free of tip cells, and assessed the ability of tip-free stalks to initiate new branches. We find stalks to be fully capable of regenerating tips that express typical tip markers, with these tips going on to form epithelial trees, at high frequency. The transition from tip to stalk is therefore reversible, at least for early stages of development. This observation has major implications for models of pattern formation in branching trees, and may also be important for tissue engineering and regenerative medicine.

KEY WORDS: Kidney, Regeneration, Stem cell, Ureteric bud, Branching

INTRODUCTION

Branching morphogenesis of epithelia is a common event in mammalian organogenesis. The process forms the airways of the lung, the milk ducts of the mammary glands, the exocrine ducts of the pancreas, the urine collecting ducts of the kidney, the seminiferous ducts of the prostate, and the ducts of salivary, lacrymal and uterine glands (Davies, 2005). Generally, these systems develop by dipodial branching, in which the ends of existing branches bifurcate and separate from one another as the tubule elongates. Although branching morphogenesis has been studied intensively for several years, significant gaps in our knowledge remain. One of the most important unanswered questions is whether the ability to initiate new branches is confined only to certain cells in a branching epithelium, for example those at the tip of an existing branch, or whether all parts of the epithelium can do it. The answer will have important implications for our basic understanding of how branched systems organize themselves and may also have implications for regenerative medicine. This report addresses this question in one of the most-studied branching epithelia, the renal collecting duct system.

The renal urinary collecting duct system arises from an initially unbranched epithelium, the ureteric bud, which invades the metanephric mesenchyme half way through mouse gestation and branches within it to produce approximately 1600 branches over approximately 10-11 rounds of bifurcation (Cebrian et al., 2004). Although much work has been done on this system, it is still not clear whether the ability to branch is confined to just a subset of cells or whether it is spread generally throughout the system: there is circumstantial evidence on both sides of the argument.

The main arguments that the ability to produce new branches is restricted to the tip concern the normal pattern of branching, the normal pattern of cell differentiation, and a close correlation between the two. Detailed time-lapse observations of renal branching morphogenesis have shown that most branching events (94%) take place by bifurcation at the ends of existing branches (Watanabe and Costantini, 2004). Cells in the terminal 70 μm of branches ('tips') are the main zone of cell proliferation (Michael and Davies, 2004) and show patterns of gene expression that differ from those in the regions behind them ('stalks'). Tip-specific markers include *Wnt11* and *Sox9*, while stalk-specific markers include collagen XVIII, *Wnt9b* and a glycoprotein that binds *Dolichos biflorus* agglutinin (DBA) (Lin et al., 2001; Michael et al., 2007; Kent et al., 1996; Carroll et al., 2005; Kispert et al., 1996). Careful measurements suggest that the zone of proliferation, the zone of *Wnt11* expression, and the zone of absence of DBA and collagen XVIII seem to respect a common boundary (Table 1). The fact that most branching takes place in the tip zone, which shows different gene expression to the stalks, suggests that there may be a tip state of differentiation that makes cells capable of initiating branches.

One of the most persuasive arguments against the ability to form new tips being restricted to existing tips is the fact that new tips appear to form from stalk regions, albeit at very low frequency and accounting for only 6% of branch events (Watanabe and Costantini, 2004). The low frequency of these events makes their interpretation difficult. It is known from careful analyses of mosaic organs, a few cells of which express green fluorescent protein (GFP), that some cells get 'left behind' by the tips to contribute to the stalk (Shakya et al., 2005). It is therefore possible that the very infrequent lateral branches actually arise from small groups of such tip cells that have not yet differentiated into stalks. A second, circumstantial, argument, comes from the fact that cell lines from renal collecting ducts can

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Table 1. Evidence that tip and stalk markers described to date respect a common boundary

Marker*	Expressed in	Boundary position	Source of information
DBA binding	Stalk	67 μm (σ=18 μm)	Michael et al., 2006
Collagen XVIII	Stalk	70 μm (σ=25 μm)	Our measurements from figure 1F,H of Lin et al., 2001
Wnt11	Tip	71 μm (σ=18 μm)	Michael et al., 2006
Sox9	Tip	89 μm (σ=38 μm)	Our measurements from figure 6A of Kent et al. 1996
High proliferation	Tip	Within the first 100 μm (low spatial resolution data)	Michael and Davies, 2004

*Some markers (Wnt9b) are missing from this list because no scale bars were provided in the micrographs presented by the authors who described their expression patterns.

produce branching tubules in three-dimensional culture systems without – as far as is known – requiring branch-producing cells to be in a separate state of differentiation (Santos and Nigam, 1993). A third argument is that various physical models of branching morphogenesis, such as viscous fingering, have no need for the ability to initiate branches to be restricted to specific cells (Fleury and Watanabe, 2002; Fleury et al., 2004). A fourth possible argument is that the Wolffian duct, from which the ureteric bud normally emerges as a single side branch, can be induced to produce supernumerary side branches by the focal application of ramogens, such as GDNF (Sainio et al., 1997; Davies et al., 1999). The problem with this argument is that the production of a side branch is an essential property of the amniote Wolffian duct, so extra side-branching from it does not necessarily imply that side-branching is a normal ability of the ureteric bud itself.

Establishing whether the ability to initiate branching is restricted or distributed within the ureteric bud/collecting duct system is important, because it carries major implications for understading patterning mechanisms and for creating strategies to promote regeneration. We have therefore directly tested the ability of stalk regions to generate new branching tips. Our results support a model in which the ability to initiate branches is distributed widely, and not restricted to cells that already express genetic markers characteristic of branch tips.

MATERIALS AND METHODS

Dissection and organ culture

Metanephric rudiments were dissected from E11.5-E17.5 CD1 mouse embryos, the ureteric bud being cut close to its junction with the Wolffian duct/bladder. Ureteric 'stalks' were removed from tip regions by cutting just below the 'T' junction of E11.5 kidneys, and the remaining tip regions were retained for staining for Wnt11 or with *Dolichos biflorus* agglutinin (DBA). Deliberate injuries to ureteric bud stalks or mesenchyme, for the experiments that needed them, were achieved by stabbing with 0.5×16-mm needles. Where surrounding stroma had to be removed from ureters (see main text), this was achieved by trypsinization in 2× trypsin-EDTA for two minutes followed by manual separation of the stroma and stalk. Organs were cultured on Isopore filters (Millipore) on Trowell-type grids in 35-mm petri dishes in MEM (Sigma M5650), with 10% fetal calf serum and penicillin-streptomycin solution in 5% CO_2 at 37°C.

RT-PCR for *Wnt 11*

For determination of the maximum possible extent of contamination of stalk numbers by tip cells, we used conventional end-point PCR to detect *Wnt11* in various dilutions of kidney cDNA that represented known numbers of tip cells. In this way, we established that we could detect *Wnt11* cDNA derived from as few as 0.81±0.1 tip cells clearly (and very faintly from reactions from smaller numbers of cells). At the same time, we used the same PCR technique (described below) to attempt to detect *Wnt11* from stalk-derived cDNA without dilution, and showed the signal in a reaction representing cDNA from 0.44 stalks (see below) to be barely detectable. This was used to conclude that 0.44 stalks included fewer than 0.81 contaminating tip cells, or that a stalk contained fewer than two contaminating tip cells.

In detail, total RNA was isolated from 28 whole kidneys, or from 35 stalks-plus-surrounding mesenchyme, using the SV total RNA isolation kit (Promega), and 200 ng of each type of RNA was used to make cDNA using the MLV-RT kit (Promega). One twentieth of the cDNA was then used for each normal PCR reaction. The actual volumes and dilutions of each stage were recorded accurately for subsequent calculations of the number of tip cells and stalks represented in PCR reactions (these calculations also used the fact that each tip consists of 117±18 cells, the measurement of which is described in the immunofluorescence section below). Tracking the dilutions of the samples as they were processed indicated that each PCR reaction from stalk cDNA represented the RNA of about 0.44 stalks and that each normal PCR reaction from kidney included RNA from a mean of 81±12 tip cells (together with many non-tip cells). Primers for β-actin were used in the normal PCR reactions to provide a further check that the dilutions used to create the normal stalk and kidney PCRs were correct and represented the same total number of cells. In addition to standard PCR reactions, reactions were also performed in which the kidney cDNA from the reverse transcription (RT) step was diluted 1/10, 1/100, 1/500, 1/1000 and 1/5000: these therefore represented RNA from 8.1, 0.81, 0.16, 0.081 and 0.016 tip cells. This dilution series was run in lanes adjacent to the normal PCRs from kidney and stalk to establish a threshold of clear detection.

For detection of *Wnt11* expression in tips growing from ureteric bud stalks, total RNA was isolated from four stalks that had been allowed to generate new tips by surrounding them with fresh E11.5 mesenchyme, and cDNA was synthesized using the same techniques and concentrations as are described above.

Fixation and immuno/lectin-fluorescence

Kidneys/recombinants intended for immuno- or lectin-fluorescence were fixed in methanol, washed in PBS with 4% milk powder and incubated in 1/100 mouse anti-calbindin-D_{28k} (Abcam) and/or 1/200 rabbit anti-laminin (Sigma) in PBS overnight at 4°C. They were then washed in PBS, and transferred to donkey anti-mouse IgG-Texas Red (Abcam) diluted 1:100 and lectin from *Dolichos biflorus* (horse gram)-FITC (Sigma) diluted 10 ng/ml (1:100 of 1 mg/ml PBS stock) or 1/100 FITC anti-rabbit (Sigma) in 4% milk powder in PBS overnight at 4°C. A final wash for 30 minutes was carried out in PBS at room temperature while agitating gently. For determination of the mean number of cells in a tip, staining with *Dolichos biflorus* lectin was used to define (negatively) the tip, as described by Michael et al. (Michael et al., 2007), and confocal microscopy was used to measure the mean volume of the cellular part of a tip ($4.8\times10^4\pm6\times10^3$ μm^3) and the mean volume of tip cells (413±37 μm^3). The ratio was used to determine the mean number of cells per tip (117±18).

Culture of stalks in Matrigel

Culture in Matrigel was performed according to the methods of Sakurai et al. and Qiao et al. (Sakurai et al., 2001; Qiao et al., 1999). Briefly, stalks were isolated and cultured in a 1:1 mix of Growth Factor Reduced Matrigel (BD Biosciences) and kidney culture medium with 125 ng/ml recombinant human GDNF (Promega), 250 ng/ml recombinant human FGF1 (R&D Systems) and 625 ng/ml recombinant human pleiotrophin (R&D Systems). The stalks were cultured for 144 hours, fixed for two hours in 4% paraformaldehyde in PBS (pH 7.0), washed in 1% Triton X-100 in PBS for 30 minutes, stained overnight in FITC-phalloidin (Sigma P5282) at 4°C and washed in PBS for 1 hour at room temperature.

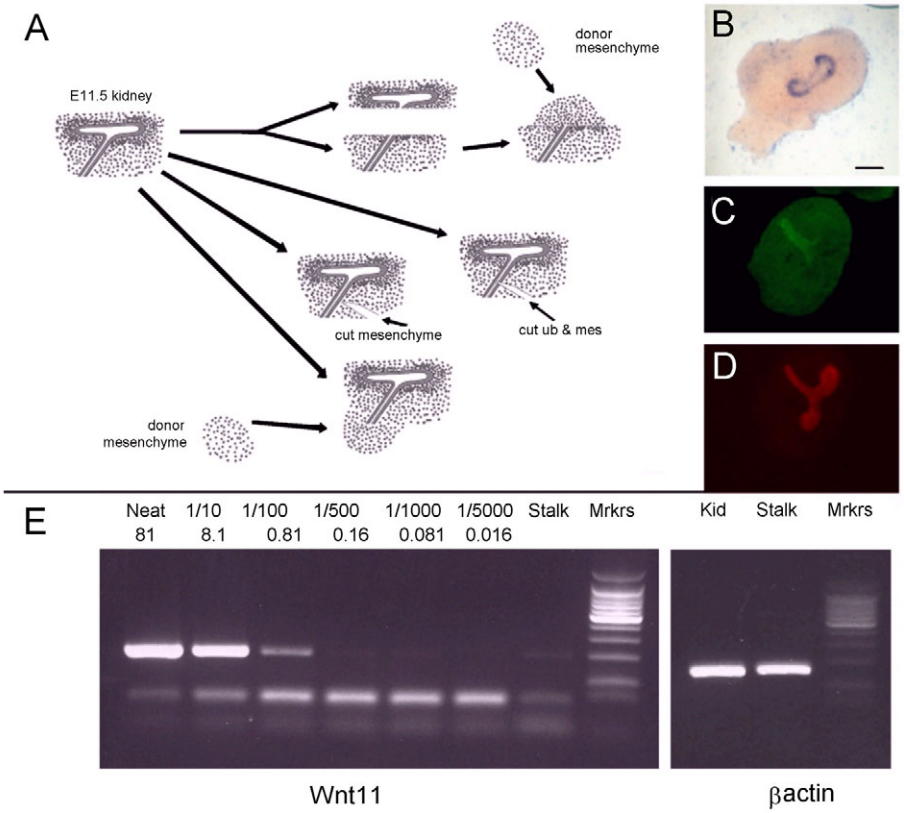

Fig. 1. Dissections and recombinations. (**A**) Diagram of the tissue manipulations used for these experiments. The top arrows indicate separation of tips and stalk, and the culture of the amputated stalk with fresh mesenchyme, the middle arrows indicate injury to mesenchyme (mes) alone, or to stalk (ub) and mesenchyme, and the bottom arrows indicate culture of the 'wrong' end of the stalk with fresh mesenchyme. (**B-D**) Discarded portions stained with an in situ probe show the complete *Wnt11*-expressing tip regions (B), in addition to short regions of *Wnt11*-negative stalks; those stained with the stalk-specific stain DBA again show that the tips and a short length of stalk are present in the discarded region (C). Staining the same specimen with calbindin-$D28_K$, which stains both tips and stalks (D), shows where the tips are. Scale bar: 100 μm. (**E**) Dilution PCR analysis of *Wnt11* expression in kidneys, including tips, and in de-tipped stalks. The numbers below the dilutions are the number of tip cells represented in the PCR. The signal in de-tipped stalks is far dimmer than the 1/100 dilution of kidney (with 0.81 tip cells). The actin bands demonstrate that the undiluted samples of kidney and stalk cDNA represent equal amounts of total cells, as intended.

In situ hybridization

The plasmid used to generate *Wnt11* probes for in situ hybridization has been used elsewhere (Kispert et al., 1996) and was kindly donated by S. Vainio. It consisted of a 2.1 kb cDNA of *Wnt11* in pSKII. Antisense DIG-labelled probes were generated by cutting the plasmid with *Xho*I and using T3 RNA polymerase; sense 'probes' were generated by cutting the plasmid with *Xba*I and using T7 polymerase. Cultures were first fixed in cold methanol to enhance their adhesion to their filters, then fixed overnight in 4% paraformaldehyde in PBS, incubated in 0.1% Tween 20 in PBS ('PBT') for 10 minutes, treated with 10 μg/ml proteinase K in PBT for 15 minutes at room temperature, washed for 3×5 minutes in PBT and post-fixed for 40 minutes in 4% formaldehyde in PBT. They were then incubated for 2-4 hours at 65°C in 50% deionized formamide, 25% 20×SSC, 2% Roche blocking powder, 0.1% Tween 20, 0.5% CHAPS, 1 mg/ml yeast tRNA, 0.5 M EDTA and 0.05% heparin. Probe, pre-heated to 80°C for 3 minutes, was added at 250 ng/ml and left overnight at 60°C. Samples were then washed in post-hybridization solution (50% formamide, 25% 20×SSC, 0.1% Tween 20, 0.5% CHAPS) for 2×10 minutes, then in 75% post-hybridization solution (2×SSC), then in 50%, then in 25%, each for 10 minutes. They were then washed in 2×SSC, 0.1% CHAPS for 2×30 minutes, and 0.2×SSC, 0.1% CHAPS for the same amount of time. They were then blocked in TBST with 10% sheep serum, incubated overnight in 1:200 alkaline phosphatase-conjugated anti-DIG (Roche) and developed the next day with NBT/BCIP solution. All buffer solutions used for in situ hybridization were treated with diethyl pyrocarbonate, and ProtectRNA (Sigma) was used in all solutions after proteinse K digestion. Sense controls were performed to support antisense experiments, and were negative.

RESULTS AND DISCUSSION

De-tipped ureteric bud stalks regenerate tips and undergo branching

In principle, ureteric bud stalks may refrain from producing new tips because they are intrinsically incapable of doing so, because they are inhibited by existing tips, or because the mesenchyme surrounding them has been rendered unsupportive of branching by the previous passage of the tip. To test the intrinsic ability of stalks to produce new tips, we removed them from the influence of existing tips, by amputating those tips, and we provided fresh mesenchyme (Fig. 1A). To confirm that the entire tip region had been removed, each amputated tip region was stained either for *Wnt11* mRNA or with fluorescent DBA, to ensure that it contained the tip-stalk boundary (Fig. 1B-D). These are the same markers that we have previously used to study stalk/tip boundaries (Michael et al., 2007), and they define the tip with much more precision than other alleged tip markers, such as Ret and Ros, as explained by Michael et al. (Michael et al., 2007). In any (rare) case that complete removal of the tip could not be confirmed, the corresponding stalk was discarded. To ensure that the fresh mesenchymes did not contain ureteric tips, they were used only if a complete ureteric bud could be recovered from the donor kidney. As an additional check on the efficiency of dissection, samples of mesenchyme were also stained with anti-calbindin-D_{28K}, a marker for ureteric buds (Davies, 1994); they were negative, as expected.

As an additional check that stalks meeting the above criteria for purity really were free of contaminating cells, a dilution-series RT-PCR was performed to set an upper limit on the possible number of tip cells that could be present in an allegedly pure stalk sample. The details of the RT-PCR and the calculations made from it are explained in the Materials and methods. It showed that *Wnt11* in as few as 0.81±0.12 tip cells, represented by the 1/100 dilution of kidney cDNA in Fig. 1E, could be detected clearly. The *Wnt11* in a PCR reaction representing the undiluted cDNA from 0.44 stalks shows a barely detectable band (Fig. 1E). Therefore, each stalk was contaminated by fewer than 0.81/0.44=1.8 tip cells. This is far fewer than those needed to make even one tip (117±18 cells), even after a few cell cycles. These PCR data

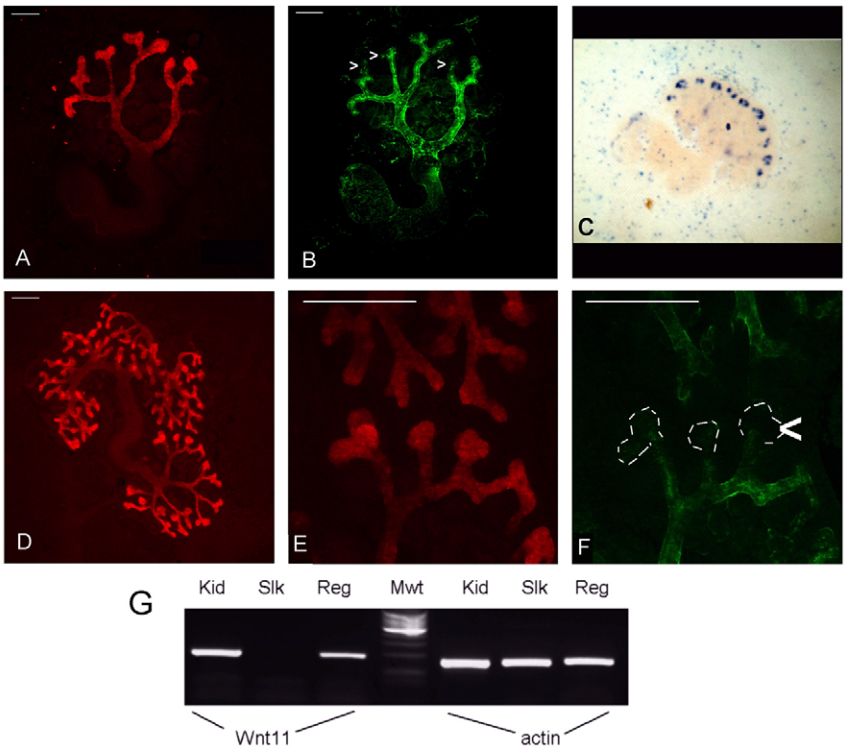

Fig. 2. Regeneration of new branching epithelia from E11.5 ureteric bud stalks. (**A**,**B**) New branching tree generated from an isolated E11.5 stalk surrounded by fresh mesenchyme, stained with (A) anti-calbindin-D_{28K} and (B) the stalk marker DBA. Comparison of the images reveals that at least some of the new tips are DBA negative (arrowheads). (**C**) Another example, stained for *Wnt11* by in situ hybridization; the new tips have acquired *Wnt11* expression. (**D-F**) Generation of a new ureteric bud tree from the 'wrong end' of the ureteric bud. (D) Low-power view of a 'double-ended' kidney formed by branching from the cut end of the ureteric bud. (E,F) Higher-power view of the 'wrong end' tree, stained with anti-calbindin-D_{28K} (E) and DBA (F): a significant number of tips (outlined in F) have greatly reduced DBA expression compared with stalk (arrowhead). (**G**) These stalk-derived tips (Reg) express the tip marker *Wnt11* by RT-PCR at levels similar to those in whole kidneys (kid), whereas the stalks not allowed to generate new tips (Slk) do not express it at detectable levels. Scale bars: 100 μm.

therefore support the in situ hybridization and immunostaining data in the paragraph above, and suggest that the stalks are not significantly contaminated by tip cells.

The majority (71%) of de-tipped stalks provided with fresh mesenchyme produced branched epithelial trees of an appearance broadly similar to those of a normal ureteric bud, albeit smaller (Fig. 2A). This fraction is approximately twice the number of stalks that could have contained even one contaminating tip cell, as calculated in the paragraph above, so cannot be due to contamination by tip cells. The other 29% simply expanded in a cyst-like manner, probably because they were damaged during handling. The branches produced in the 71% of cultures that produced trees terminated in ampulla-shaped tips that were indistinguishable from those of normal kidneys. What is more, in situ hybridization and RT-PCR for *Wnt11* showed that this tip marker was expressed at the tips of the branches generated by the stalk (Fig. 2C,G), and DBA lectin staining showed that this stalk marker was reduced in or absent from most new tips (Fig. 2B). The de-tipped stalks were therefore capable of regenerating tips that had normal marker expression, as well as normal morphology. The proportion in which this occurred, just over 70%, is much higher than the 6% of branches that seem to arise laterally from stalks in normal renal development (Watanabe and Costantini, 2004), and it is not reasonable to assume that these could have arisen from 'lost' clusters of tip cells left behind: if there were that many 'lost' tip cells, we and others would have seen them in *Wnt11* in situ stains.

Branching and tip formation can be induced even from the wrong end of the ureteric bud

To determine whether the ability to initiate branches was still present even in the most distal regions of the ureteric bud stalk, we left the existing tips of ureteric buds alone and instead packed fresh mesenchyme around the distal end of the ureter that was severed when the kidney was isolated from the embryo (Fig. 1A). Forty percent of the E11.5 kidneys so treated showed prolific branching from the severed ureter to produce 'double-ended' trees (Fig. 2D). These tips lost DBA-binding activity (Fig. 2E,F) and also induced the formation of nephrons in the surrounding mesenchyme (Fig. 3C,D). This ability is retained by ureters from both E11.5 and E12.5 kidneys (Fig. 3A).

These results demonstrate that the ability of the ureteric bud to initiate new branch tips is not restricted to existing tips but is instead distributed widely, at least for the first few days of the bud's existence. This possibility has been suspected recently from time-lapse studies of ureteric branching (Shakya et al., 2005; Watanabe and Costantini, 2004), but, as pointed out in the recent review of Costantini and Shakya, it has not been directly examined before (Costantini and Shakya, 2006). The finding also implies that the specialized state of gene expression at the tips (Wnt11-positive, DBA-negative, etc) might be required for the proper organization of branching morphogenesis, but it cannot be needed for cells to make their first response to ramogenic signals. If it were, the $Wnt11^{-}$, DBA^{+} stalks could not have responded. Expression of molecules such as Wnt11 must therefore be secondary to the events that first induce new branches to form.

Although the distal ends of ureters of E11.5 and E12.5 kidneys could produce new branches when provided with fresh mesenchyme, those of E13.5, E14.5 and E15.5 kidneys failed to do so. These epithelia are surrounded by a sleeve of stroma that might, conceivably, inhibit tip formation. To address this possibility, we removed the stroma enzymatically before applying fresh E11.5 mesenchyme to the ureter epithelium. It was possible to remove 100% of stromal cells from ureters up to and including E13.5, but from E14.5 only about 90% of the cells could be removed (leaving significant uncovered areas of epithelium); further extending the enzymatic incubations resulted in the tissue losing structure completely. The E13.5 ureters freed completely from stroma were able to produce new tips when

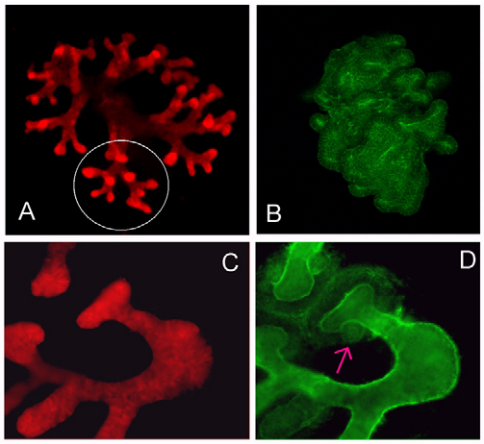

Fig. 3. Generation of branched ureteric systems from the 'wrong end' of the ureters of more mature kidneys. (**A**) A cut ureteric stalk of an E12.5 kidney capped with E11.5 metanephric mesenchyme ramified through the mesenchyme to generate a branched collecting duct system (circled). (**B**) Branching morphogenesis of an E11.5 de-tipped stalk transferred to matrigel with GDNF, FGF1 and pleiotrophin, incubated for 144 hours and stained with FITC-phalloidin to reveal its anatomy. It is interesting to note that the phalloidin stain is particularly strong in the apical regions of cells at the branch tips, as described for normal ureteric buds developing in whole kidneys (Michael et al., 2005). (**C**,**D**) Tips formed from the 'wrong end' of the ureter, as in A, induce the formation of nephrons in the surrounding mesenchyme. These are not detectable in the ureteric bud-specific anti-calbindin stain (C) but are visible in the anti-laminin stain (D, arrow).

provided with fresh E11.5 mesenchyme, and these tips went on to induce nephrons in that mesenchyme, suggesting that the failure of E13.5 ureters surrounded by stroma to produce new tips was due to an inhibitory influence of the stroma. Later ureters that could be freed substantially but not completely from stroma still failed to form tips. A simple mechanical influence of stroma, for example that it forms a diffusion barrier to molecules such as GDNF from the fresh mesenchyme outside it, is unlikely to explain this effect, as even the older enzyme-treated ureters had lost enough stroma to make the epithelium accessible. Bmps such as Bmp4 and Bmp5 are expressed strongly in this stroma (Dudley and Robertson, 1997), and are known to be inhibitors of branching (Hartwig et al., 2008; Gupta et al., 1999; Miyazaki et al., 2000; Michos et al., 2007). Gremlin 1 is a powerful antagonist of Bmps, particularly Bmp2 and Bmp4, and treatment of cultured kidney rudiments with exogenous gremlin 1 is sufficient to antagonize Bmp activity and alter ureteric branching in intact kidneys (Michos et al., 2007). To test whether the secretion of Bmps by the remaining peri-ureteric stroma might account for the repression of tip formation in our system, we applied the Bmp antagonist gremlin 1 at 5 μg/ml to the cultures. This concentration was the same as that used by Michos et al., and, in our hands, it had a modest effect on increasing the amount of branching in E11.5 kidneys, by 14% (P=0.073), suggesting that the molecule was active. It failed, however, to induce tip formation from the enzyme-treated E14.5 ureter/ fresh mesenchyme combinations. This suggests that the stroma secretes an inhibitor other than Bmps, or that the ability to produce new tips is lost as the epithelium itself matures.

New tip formation is a response to fresh mesenchyme, not to tissue injury

The process of setting up the cultures described above necessarily involved cutting mesenchymal and epithelial tissues. It was therefore possible that the production of new branches was simply a response to injury. To test this, two types of cutting experiment were performed without any transplantation of mesenchyme. In the first, a syringe needle was used to cut a slit in the mesenchyme adjacent to one side of the ureteric bud stalk but with no injury to the stalk itself and in the second, the cut passed through the stalk itself, as well as the surrounding mesenchyme (Fig. 1A). The injured kidney rudiments were then incubated for 6 days, uninjured kidneys being used as controls. None of the kidneys in either control or cut groups showed any evidence of branching from the stalk. Conversely, when mesenchyme was removed from the side of the stalk of the ureteric bud, without injuring the bud itself, and replaced by a clump of fresh metanephric mesenchyme, 75% of kidneys demonstrated emission of new branches from the side of the stalk. Injury alone was not therefore a sufficient trigger for production of new tips; fresh mesenchyme was required.

De-tipped stalks branch when placed in a three-dimensional matrix

Intact ureteric buds will grow and branch when placed in a three-dimensional gel matrix, consisting of Matrigel supplemented with GDNF, FGF1 and pleiotrophin (Sakurai et al., 2001). Isolated, de-tipped stalks transferred to this culture system, grow and branch in a manner similar to that of intact ureteric buds (Fig. 3B). This demonstrates that ramogenic factors already characterized in normal mesenchyme (GDNF, FGF1 and pleiotrophin) are sufficient to promote the regeneration of tips. It is notable that the density of tips is much higher in this system than in normal kidneys.

Ureteric stalks, then, are capable of forming new tips if provided with fresh mesenchyme or with a Matrigel artificially loaded with ramogens, such as GDNF and FGF1, known to be manufactured by fresh mesenchyme (Sainio et al., 1997; Sakurai et al., 2001). It is known that GDNF is not expressed by mesenchyme cells after they have been induced, by contact with the ureteric bud, to form neprhons and stroma (Sainio et al., 1997). FGF1 persists a little longer, but is still lost as nephrons mature beyond the 'S'-shaped stage (Cancilla et al., 1999). Indeed, not only do maturing nephrons and stroma cease to produce ramogens, they also begin to secrete anti-ramogenic factors, such as Bmp2 and Tgfβ (Lyons et al., 1995; Ritvos et al., 1995; Davies and Fisher, 2002; Dudley and Robertson, 1997; Bush et al., 2004; Gupta et al., 1999; Piscione et al., 1997). This suggests a model in which stalks are normally prevented from branching because the mesenchyme that surrrounds them has already ceased to express ramogens. The likely importance of the mesenchyme in modulating the production of tips by the stalks is supported by the behaviour of stalks in ramogen-enriched Matrigel. The density of tips formed by the stalk is much higher than that seen in normal kidney development, suggesting that in the normal organ the mesenchyme surrounding the stalk must be non-permissive for tip formation. Indeed, it is the source of factors, such as heregulin α (neuregulin 1 – Mouse Genome Informatics), that support growth and maturation of the bud without inducing branching (Sakurai et al., 2005).

This system described above would, under normal circumstances, tend to restrict branching to the existing tips because these are the only cells that meet uninduced mesenchyme. Only if mesenchymal cell mixing, and/or inefficient branching of the bud throughout the mesenchyme, brought a population of uninduced mesenchyme cells

near to a stalk would production of a new tip by the stalk occur. A system organized according to these principles would be robust against errors, because any zones of the kidney 'missed out' by the branching of the tree would be able to induce secondary branches from stalks until they were adequately served. This presumably accounts for the very low, but non-zero (6%), frequency with which lateral branches have been observed to occur in culture (Watanabe and Costantini, 2004).

Understanding that the whole of the ureteric bud is capable of producing a branching tree, at least until it has matured too far, may have implications beyond the need to revise models for the control of pattern formation in this system. There is increasing interest in using the techniques of stem cell biology and tissue engineering to repair kidneys made defective by congenital disease or infection (Hayashi, 2006; Rookmaaker et al., 2004). Most current effort is aimed at using transplanted progenitor cells to create areas of kidney in which new nephrons, free of genetic defects, develop. The absence, in a fully formed kidney, of active ureteric bud tips to provide these areas with a collecting duct system has been seen as a potential problem of the technique. If, however, the stalks of the cortical bud/collecting duct system can generate new tips anyway, either at once or as a result of minor treatment, the entire enterprise becomes much more hopeful. For this reason, our observation that stalks can regenerate tips may have implications for regenerative medicine, as well as for basic developmental biology.

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Appendix 2

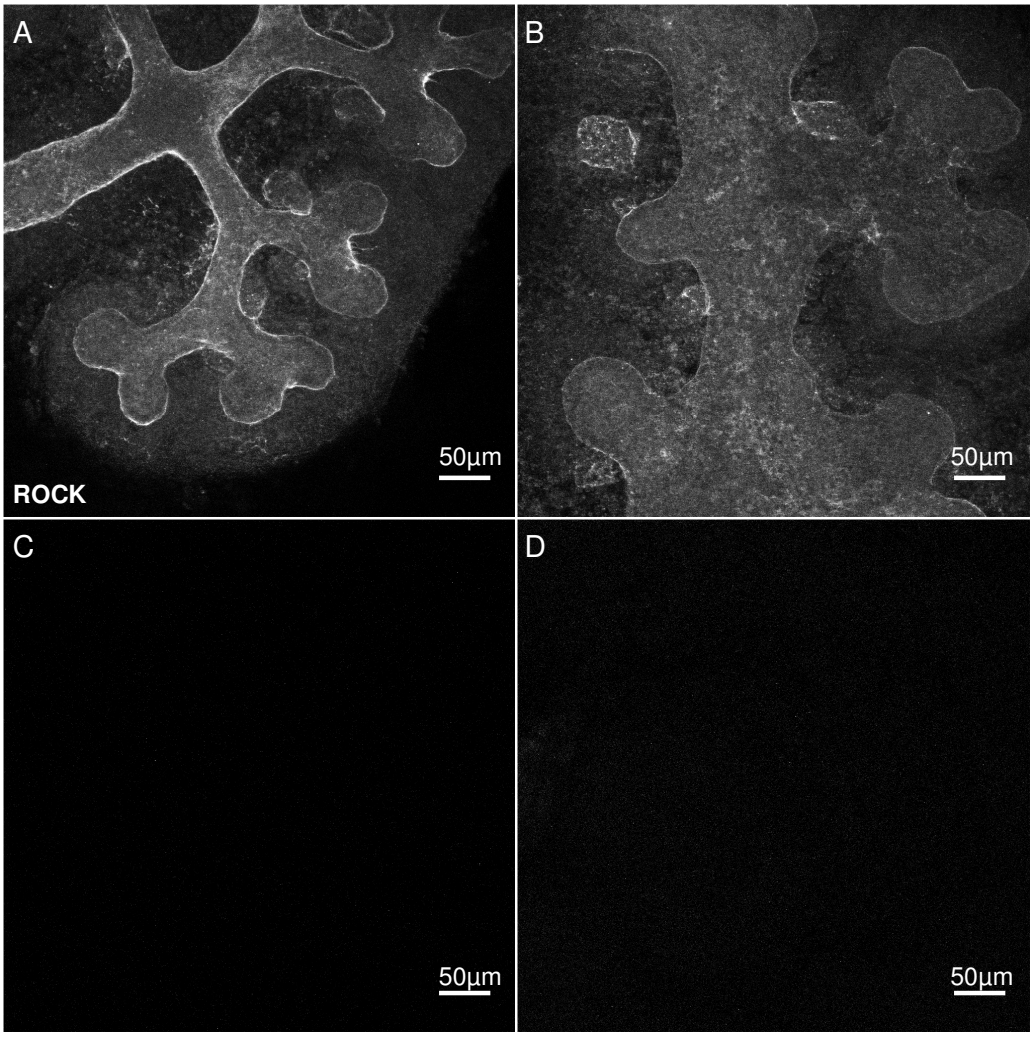


Fig.A2.1 Localisation of ROCK-II in cultured kidneys

Fig.A2.1 shows kidneys that were cultured in CKCM for 72hrs prior to fixation in methanol. Cultures were stained for ROCK using mouse anti-ROCK-II. This was detected using anti-mouse-FITC. Antibody controls were performed using primary antibodies only (C) or secondary antibodies only (D). (A) and (B) show cultures stained with both primary and secondary antibodies; these cultures differ in that they were culture without or with 1.25µM H1152, respectively.

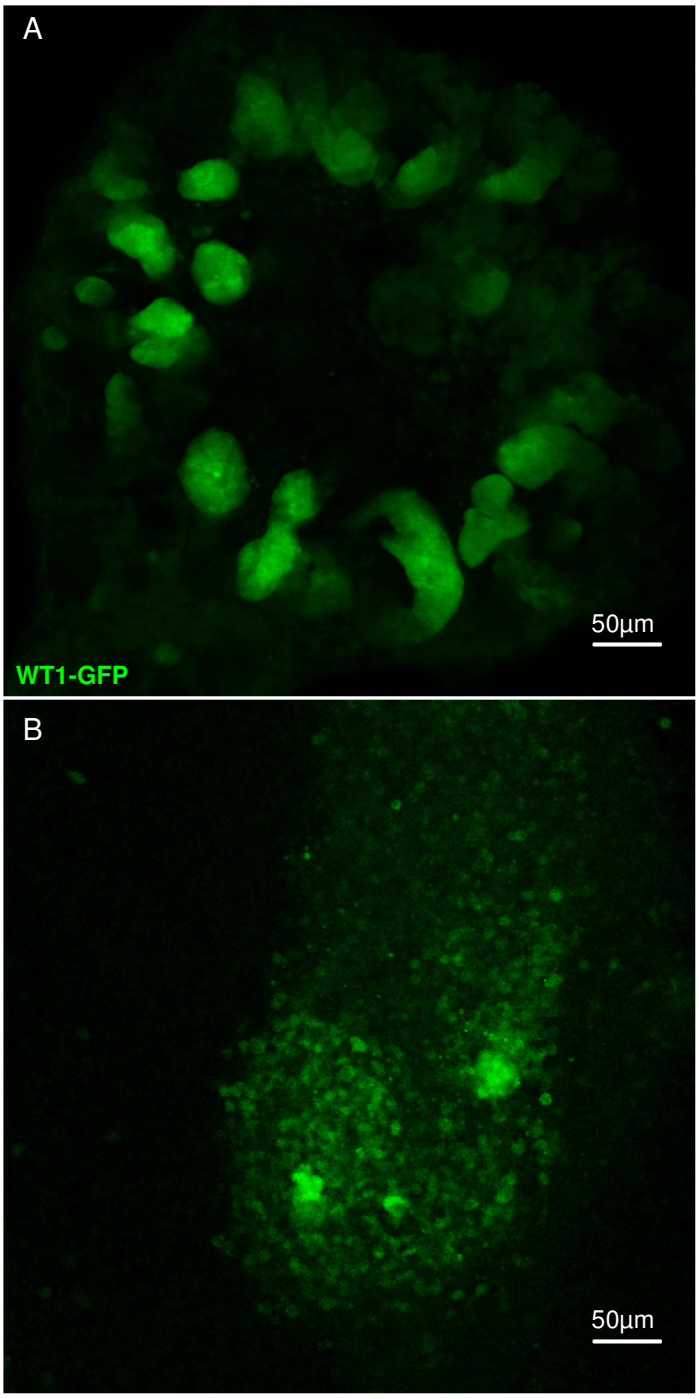
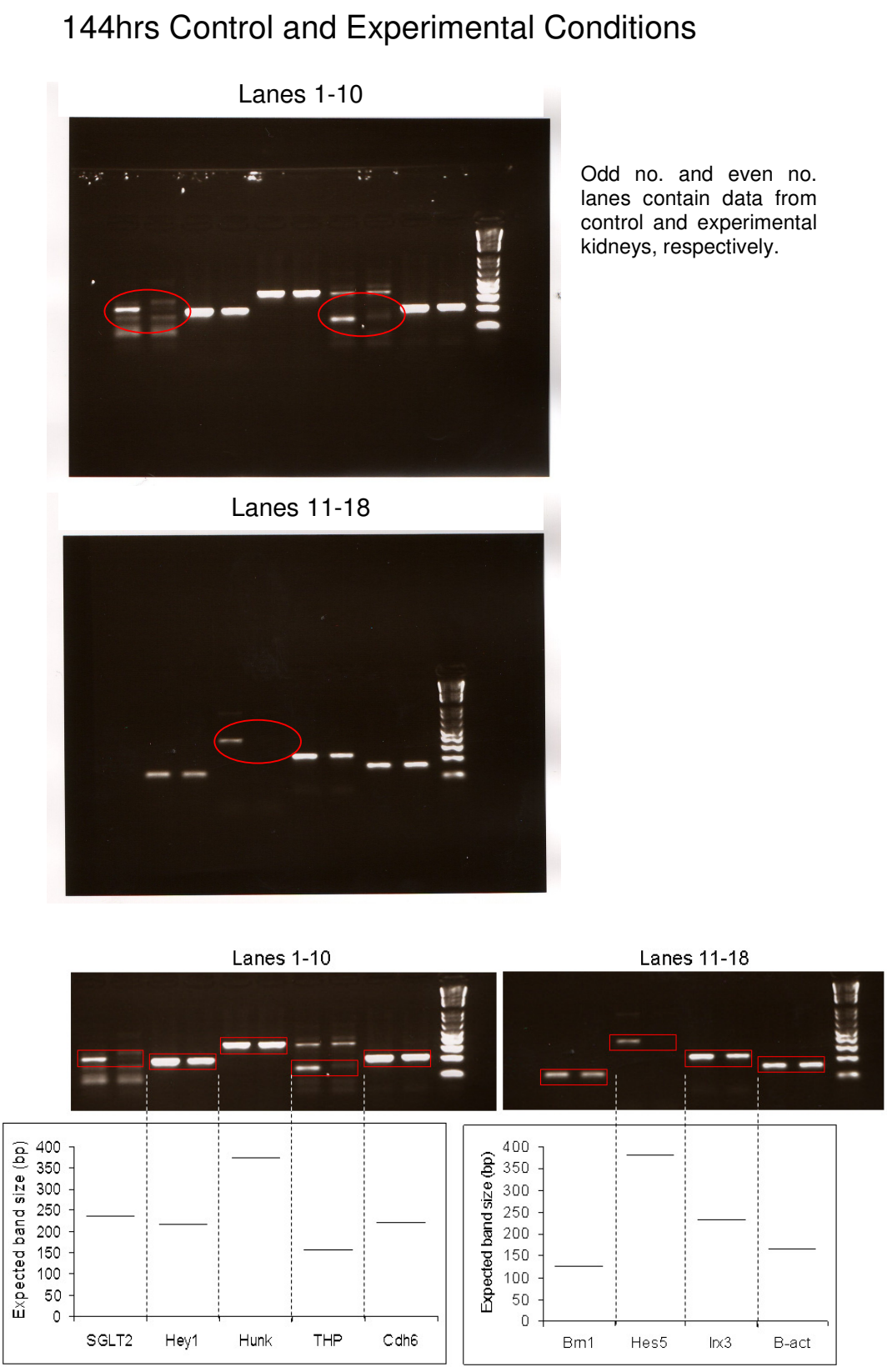


Fig.A2.2 The effects ROCK inhibition on nephron formation as viewed using WT1-GFP

Fig.A2.2 shows MM from a mouse strain heterozygously carrying a knock in of GFP into the endogenous WT1 locus, which was separated from the UB and induced to form nephrons using spinal

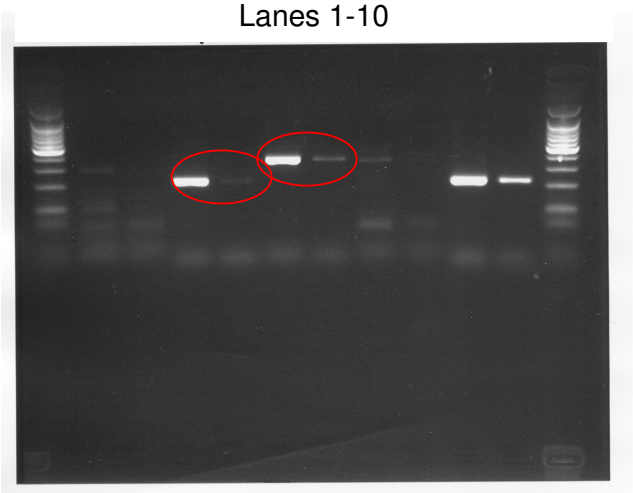
cord. Cultures were placed in CKCM without (A) or with (B) 20μM Y27632. Cultures were kept for 72hrs prior direct visualisation of the WT1-GFP protein.

Fig.A2.3 Original gel-images for data presented in Chapter 4: Fig.4.11.

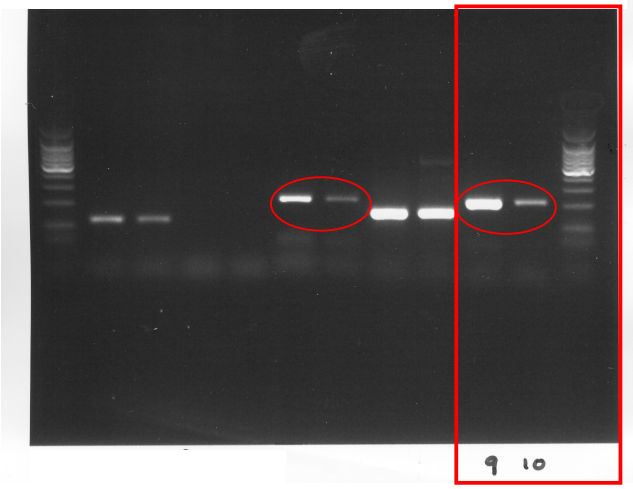


96hrs Control and Experimental Conditions

Lanes 1-10



Lanes 11-18

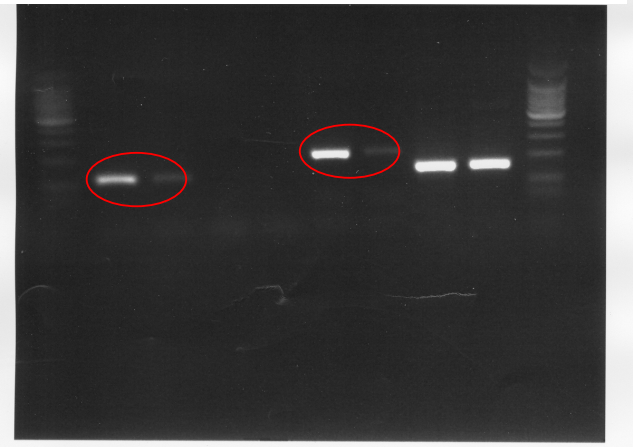


These lanes display the expression of cdh-6 in control and experimental conditions for cultures maintained for 72hrs.

72hrs Control and Experimental Conditions

Lanes 1-10

Lanes 11-18



The Cdh-6 data is shown in lanes 17 and 18 on the gel displaying the expression profiles for cultures kept for 96hrs.

24hrs and 48hrs Control Conditions

Lanes 1-10

Lanes 11-18

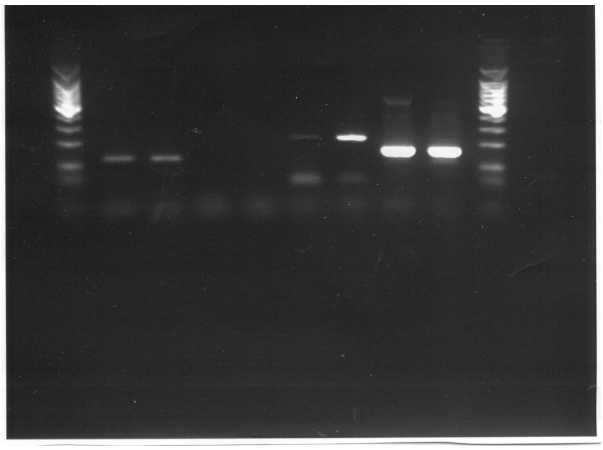


Fig.A2.3 Original gel-images for data presented in Chapter 4: Fig.4.11.

Fig.A2.3 shows original gel images for “144hrs Control and Experimental Conditions” “96hrs Control and Experimental Conditions”, “72hrs Control and Experimental Conditions” and “24hrs and 48hrs Control Conditions”. All lanes at each time-point are as specified for the “144hrs Control and Experimental Conditions” images, unless specified otherwise. Odd and even numbered lanes contain cDNA produced from control and experimental data, respectively. For “24hrs and 48hrs Control Conditions”, odd and even numbered lanes contain cDNA produced from kidneys cultured for 24hrs and 48hrs, respectively.